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CHOLESTEROL CHOLELITHIASIS - ROLE OF DIETARY FIBER

By

© RODERICK McDOUGALL

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"And the fruit of righteousness is sown in peace of them that make peace".

James 3.18

TO

ALL THOSE WHO HAVE SUFFERED, OR WILL SUFFER FROM

CHOLESTEROL GALLSTONES

ABSTRACT

Epidemiological studies have indicated that cholesterol gallstones may be related to a deficiency in dietary fiber. It has been shown that the major abnormality in gallstone patients is supersaturation of their bile with cholesterol. Cholesterol solubility depends on its incorporation into mixed micelles with bile acids and phospholipids. Total bile acid pool size is reduced in gallstone patients and there is some suggestion that the bile acid composition of the bile acid pool is also different from normal.

In this investigation three studies were made. Firstly, bile acid composition of bile was compared between eight gallstone patients and six patients without biliary tract disease. Secondly, the effect of a 50 gm per day dietary supplement of All Bran on biliary lipid composition, bile acid pools and cholesterol transport (lipoproteins) was investigated in nine gallstone patients and nine normal volunteers. Thirdly, a study was made of the effect of bran supplement on fecal weight, fecal bile acid, and neutral sterol output in four normal volunteers.

In the first study, the secondary bile acid, deoxycholic acid (DCA), was found to be significantly increased in the bile of gallstone patients, suggesting that the colonic phase of the entero-hepatic circulation was altered in these patients. In the second study, comparison between the gallstone patients and volunteers before

and after the high fiber diet produced several significant findings. DCA was again increased in the gallstone group, furthermore chenodeoxycholic acid (CDCA) was reduced. Total pool size was also reduced. The bran diet returned the cholesterol supersaturation of gallstone bile to normal and also reduced its DCA content. There was no significant change in the proportions of the other bile acids, although total pool size showed a trend to increase. High density lipoprotein cholesterol ratios were originally high in the gallstone patients. The longterm effect of the bran diet was to return the lipoprotein distribution of cholesterol towards normal. In the third study, daily fecal volume, fecal bile acid and neutral sterol excretion were shown to increase with the addition of dietary fiber (bran).

It was suggested from this study that dietary fiber deficiency offers an explanation for the geographic distribution of cholesterol gallstones, and may be responsible for their formation. Furthermore, correction of fiber deficiency should prevent their formation and may possibly, in the longterm, induce their dissolution. Dietary fiber exerts its effects on biliary lipid metabolism and cholesterol transport by increasing the daily excretion of cholesterol and bile acids.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Cholesterol cholelithiasis (gallstones) is a disease of almost plague-like proportions. It has been estimated that 10% of males and 20% of females in the 50 year age group have gallstones. The only accepted treatment of this disease is surgical removal of the gallbladder, and in Canada in 1974, approximately 80,000 of these operations were performed at a cost of well over $280,000,000. Although the majority of stones are asymptomatic, the risk of biliary colic, biliary obstruction, acute cholecystitis, ascending cholangitis, perforation of the gallbladder, carcinoma of the gallbladder and even death (about 8,000 cases yearly in the USA) is sufficient to dictate treatment of this common disease.

A more logical approach to such a common and costly disease would be to discover its etiology and direct all possible efforts at its prevention. To date, the most significant finding in regard to etiology was the discovery that cholesterol supersaturation of bile occurs in gallstone patients, and is a prerequisite for the formation of stones. In fact, gallstone dissolution can be accomplished by desaturating bile through the oral administration of certain bile acids. Obviously if bile never becomes supersaturated with cholesterol, stones should never form.

Although stasis, infection, inflammation, obesity and estrogens have all been postulated as the agents which produce cholesterol supersaturation, improper diet may be the most likely explanation.

Studies by Dennis Burkitt on the worldwide distribution of gallstones led him to believe that they are a disease of fiber deficiency. He observed that cholesterol gallstones are a rarity in rural Africa as opposed to their frequency in North America, Europe and other industrialized areas of the world. Correlating this information to the obvious difference in the fiber content of the natural diet that the rural Africans consumed, Burkitt hypothesized that in some way fiber deficiency is responsible for the formation of cholesterol gallstones.

The well known effect of a high fiber (bran) diet is to increase fecal bulk and reduce intestinal transit time. Bran is a natural laxative and is marketed by North American and European cereal companies as such. It is therefore possible that its inclusion in the diet could affect both cholesterol and bile acid metabolism, since their main elimination from the human body is in the feces. Interestingly, the bile acids, which are the major catabolites of cholesterol, are bound to a component of vegetable fiber identified as lignin.

This information has suggested that a thorough biochemical investigation of the effects of a high fiber diet on bile acid and cholesterol metabolism could be of more than academic interest. Since the obvious effect of fiber deficiency is to slow intestinal (especially colonic) transit time, and it is known that the secondary bile acids (deoxycholic and lithocholic acids) are produced in the colon, bile acid fractionation was compared in gallstone and non-gallstone patients.

After the encouraging results from this initial study

suggesting that there is an alteration in the colonic phase of the enterohepatic circulation of bile acids in gallstone patients, an investigation comparing the effects of a high fiber diet on biliary lipid metabolism in gallstone and non-gallstone volunteers was undertaken. Having shown that a high fiber diet effectively reduces the cholesterol saturation of bile, and decreases the proportion of deoxycholic acid in the bile of gallstone patients, we investigated the effects of the same diet on the excretion of biliary lipids. Our study on fecal steroids shows that dietary fiber affects biliary lipid metabolism by increasing the elimination of cholesterol and bile acids from the body. Additionally, our investigations show that systemic cholesterol transport is favorably altered by the high fiber diet.

Fiber deficiency appears to be responsible for the formation of cholesterol gallstones. The addition of adequate amounts of fiber to the diets of industrialized nations may well reduce the suffering and cost incurred by this common disease.

LITERATURE REVIEW

INTRODUCTION

The vast majority of diseases of the liver and bile duct system are associated with cholesterol gallstones, and are among the most common afflictions of adult life. Extrapolating the statistics of the Framingham study it can be estimated that 1.5 million Canadians have gallstones.[1] In fact, the incidence of gallstones in the 55-64 age group is 10% in males and 20% in females.[2] Because of the large numbers of affected individuals, the socioeconomic impact of gallstones is impressive. In the United States, for example, surgery (at no small expense) is eventually performed in about half of the estimated 800,000 new cases of cholelithiasis each year. Between 5,000 and 8,000 people (USA) die from the complications of gallstone disease yearly. The cost of treatment and time lost from work approaches over one billion dollars annually.[3]

Although up to 50% of gallstones are asymptomatic, the risk of complications is sufficient that current surgical thinking dictates their removal. Symptoms range from vague dyspepsia through biliary colic, to the severe pain of acute cholecystitis. Complications can include obstructive jaundice and cholangitis, biliary fistulae, acute cholecystitis, gallstone ileus, peritonitis, and even death.[4] The etiology of gallstones is unknown, although it is now accepted that cholesterol supersaturation of bile is necessary for their formation.[5]

In this review of the literature, the prevalence of gallstones, their composition, theories of origin and the physico-chemical basis of formation will be reviewed. Bile acid, cholesterol, and phospholipid metabolism in relation to biliary secretion, the control of biliary secretion, and medical treatment of gallstones will also be reviewed. With this knowledge as a background the information from experiments presented in this thesis will be woven into a coherent theory in an attempt to explain the etiology of cholesterol gallstones.

PREVALENCE

The most common type of gallstone in North America is composed mainly of cholesterol and accounts for over 85% of biliary calculi. These gallstones are classified either as pure (95% cholesterol or greater), or mixed cholesterol stones (which are over 70% cholesterol by weight).[6] This abundance of cholesterol gallstones is in distinct contrast to rural Asia or Africa, where pigment stones prevail.[7] The common type of stone is changing in some countries. For example, in Japan prior to World War II calcium bilirubinate stones comprised about 70-80% of the total. Following the war, the Japanese incidence of cholesterol gallstones has risen so that only 30-40% of stones are of the pigment variety.[8] Currently calcium bilirubinate stones occur mainly in rural Japan, while cholesterol cholelithiasis appears to be a disease of cities.[9] Interestingly, transplanted Africans and Asians adopt the same incidence of cholesterol gallstones as their American Caucasian neighbours.[1] There also exist genetically isolated groups of

people with both a high and a low incidence of gallstones. An extremely high incidence of cholesterol gallstones has been found in several North American Indian tribes, such as the Pima and the Chippewa.[10 11] In contrast, the Masai tribe of Africa does not form gallstones.[12] This and other geographic data has been examined by Burkitt, who hypothesizes that gallstones are the consequence of the highly refined fiber-deficient diets so common to highly industrialized countries, such as Canada and the United States.[13]

GALLSTONE FORMATION

The traditional classification of gallstones was introduced by Aschoff in 1924, and has persisted to date. He hypothesized that stones were either:

1. Inflammatory.
2. Metabolic (pure pigment, calcium bilirubinate, or pure cholesterol).
3. Combination (primary metabolic plus secondary inflammatory)

   or
4. Stasis (primary in common duct - earthy).[14]

Such a classification is now regarded as erroneous. Since most calculi are chemically similar in composition, any classification which attempts to relate composition to etiology is somewhat unjustified.[15] It is now generally accepted that there are four major types of stones: cholesterol, calcium carbonate, calcium bilirubinate, and a rare organic material stone.[16]

The main constituents of gallstones are cholesterol

monohydrate, calcium bilirubinate, and calcium carbonate, whether the stones be "pigment" or "cholesterol".[15 17] Other substances include bile acids, fatty acids, phosphorus, iron, copper and manganese. Unconjugated bilirubin has been found in the center of stones.[19] Protein is also a component of gallstones, particularly their centers.[19] Further investigations have shown that the centers always contain a pigmented material and usually are composed of a mass of small crystals and flaky pigmented material trapped in small strands of fibers.[16] Histochemical studies have shown that this nucleus or nidus invariably contains mucopolysaccharide. Mucopolysaccharides are thought to be an important structural component of gallstones.[8 20] Cholesterol crystals are in fact arranged on a framework of mucopolysaccharide radiating from the centers of some stones.[21]

THEORIES OF ORIGIN

Traditional theories regarding the origin of gallstones are inflammation, nucleation and stasis. More recently endocrines, obesity and diet have been implicated. In the gallbladder, the role of mucus and abnormal proteins have received attention. Finally, the physicochemical basis of cholesterol supersaturation of bile in gallstone disease has been investigated. All these attempts have so far failed to explain completely the etiology of cholesterol gallstones.

It has long been held that inflammation and infection are responsible for the formation of gallstones, and explanations as to

how infectious agents reach the gallbladder are old and numerous.[22] Naunyn in 1892 proposed that mixed stones arose as a pultacious mass later invaded by cholesterol.[23] Infection may play some role in the formation of stones, either as a primary cause with clumps of dead bacteria acting as a nucleus or nidus, or as a secondary cause, resulting in stone growth.[24] More recently papillitis, ascending infection, and Ascaris infestation have been suggested as causes for the calcium bilirubinate stones in Japan.[9] β-glucuronidase, produced by various anaerobic bacteria, deconjugates bilirubin diglucuronide in bile to produce free bilirubin, which rapidly combines with biliary calcium and forms an insoluble precipitate which coalesces to produce calculi. Maki has demonstrated that virtually all Japanese with calcium bilirubinate stones have been infected with β-glucuronidase producing E. coli.[8] It has been suggested that inflammation from any cause may result in the formation of gallstones. Ostrow has shown that the inflamed gallbladder selectively absorbs the bile acids which are necessary to maintain biliary cholesterol in solution. Absorption of sufficient bile acids from the inflamed gallbladder could cause cholesterol to precipitate and ultimately result in gallstone formation.[5 25 26 27]

The nucleation theory was introduced by Boysen in 1900. He hypothesized that tiny pigment calculi can form in the intrahepatic canaliculi as a consequence of liver disease. These calculi travel with bile flow to the gallbladder where they presumably act as a nidus for cholesterol precipitation, and will ultimately produce

gallstones.[24] Numerous agents have been postulated as nuclei for cholesterol precipitation. Bacteria, mucus plugs, foreign bodies such as suture material, epithelial debris, or even parasites may be a site for cholesterol precipitation, and lead to gallstone formation.[24 28]

In 1957 Coe introduced the concept of stasis as an etiologic factor in gallstone formation. More recently, an experimentally produced incomplete stricture of the terminal common bile duct in the dog has been demonstrated to induce gallstone formation. It was theorized that prolonged stagnation allowed cholesterol to precipitate from bile.[24] Another possibility may explain these results. Biliary stasis can reduce the output of bile acids and lecithin after a short period of time, both of which are necessary for cholesterol solubility in bile. Their absence will result in cholesterol precipitation.[30]

The endocrine effects of pregnancy and female sex have also been related to cholelithiasis. Females are twice as likely to develop gallstones as men, and the incidence increases with the number of pregnancies.[1 31] A possible explanation is the demonstration of gallbladder stasis during the normal progestational phase of the menstrual cycle, and furthermore, the finding that postprandial evacuation is delayed after the fourth month of pregnancy.[32] How important this degree of stasis is, is open to question. For example, regular emptying of the gallbladder by cholecystokinin injections in rabbits on a lithogenic diet does not prevent the formation of

gallstones.[33] Additional evidence further implicates estrogens in gallstone formation. Lynn has demonstrated that estrogen administration tends to make bile lithogenic in the primate, and also reduces bile flow.[34] In the rat, bile acid synthesis is reduced during pregnancy, implying that cholesterol solubilization in bile could be impaired during pregnancy.[35] Women who use oral contraceptives in fact have 2.5 times greater risk for gallstone formation than do non-users.[36] There remains little doubt that if a person is susceptible to gallstone formation, the administration of estrogens can convert the tendency into reality.[37]

Obesity has also been related to cholelithiasis.[1, 31] Caloric intake is higher in people with gallstones; however, protein, carbohydrate and lipid composition of the diet is identical to that of people without gallstones. Interestingly, working time is lower and rest time is significantly higher in women with gallstones. Biliary cholesterol output is stimulated when caloric intake is raised.[38] Increased biliary cholesterol secretion has been demonstrated in obesity and is probably related to gallstone formation in the obese patient.[39, 40]

Diet could influence the occurrence of gallstones, as suggested by many investigators. An increase in daily protein consumption augments cholesterol synthesis, whereas triglyceride feeding has little effect on bile acid or cholesterol production. Cholesterol feeding, however, increases bile acid synthesis.[38] Glucose feeding in the rat results in a marked increase in biliary excretion of cholesterol and bile acids, indicating augmentation of synthesis.[41] Similarly,

carbohydrate feeding in man increases the lithogenicity of bile, probably by stimulating cholesterol synthesis.[42] Cholesterol feeding increases the lithogenicity of bile in several species including hamsters, ground squirrels, prairie dogs, and the squirrel monkey.[43, 44] A high level of dietary cholesterol will similarly render bile more lithogenic in man.[45] Gallstones can be produced in animals such as the hamster by dietary manipulation (high carbohydrate and high cholesterol).[46, 47] Protection from the lithogenic diet can be provided by the addition of plant fiber.[48] In fact, it is suggested by geographic studies on cholelithiasis, that lack of dietary fiber may play an important role in the genesis of cholesterol gallstones in man.[13]

Mucus may well have an important function in the development of gallstones. Pathological human bile contains more hexosamine, and is more viscous than normal gallbladder bile.[49] It has been suggested that entrapment of cholesterol crystals by gallbladder mucus allows stone formation to proceed by preventing the normal flushing action of gallbladder contraction from removing such debris.[33] An increased secretion of mucus precedes the formation of gallstones in rabbits being fed dihydroxycholesterol. This secretion is prevented if pathological bile is not allowed to enter the gallbladder.[50] Entrapment of cholesterol by mucus and crystal growth in mucus gels has been demonstrated in Syrian hamsters. A prosthesis of mucus is probably necessary for the formation of gallstones.[51]

Abnormal biliary proteins have been implicated as a possible

etiologic factor. Englert has demonstrated sixteen total biliary proteins and four bile specific proteins, using immunochemical methods. Evidence was also obtained for either a missing and/or an abnormal protein in the gallbladder bile of gallstone patients.[52] The absence of a stabilizing lipoprotein has also been postulated.[53]

Cholesterol supersaturation of bile is the main abnormality found in gallstone patients. Supersaturation is now recognized as the physicochemical basis of cholesterol gallstone formation.[5]

THE PHYSICOCHEMICAL BASIS OF GALLSTONE FORMATION

It has been known for some time that bile acids have the power to dissolve cholesterol in aqueous solutions. Cholesterol is by itself insoluble in water, and bile contains over 95% water. Aside from this slight information about the ability of bile acids to solubilize cholesterol, little data was available on the mechanics of cholesterol transport in bile prior to 1930.[25]

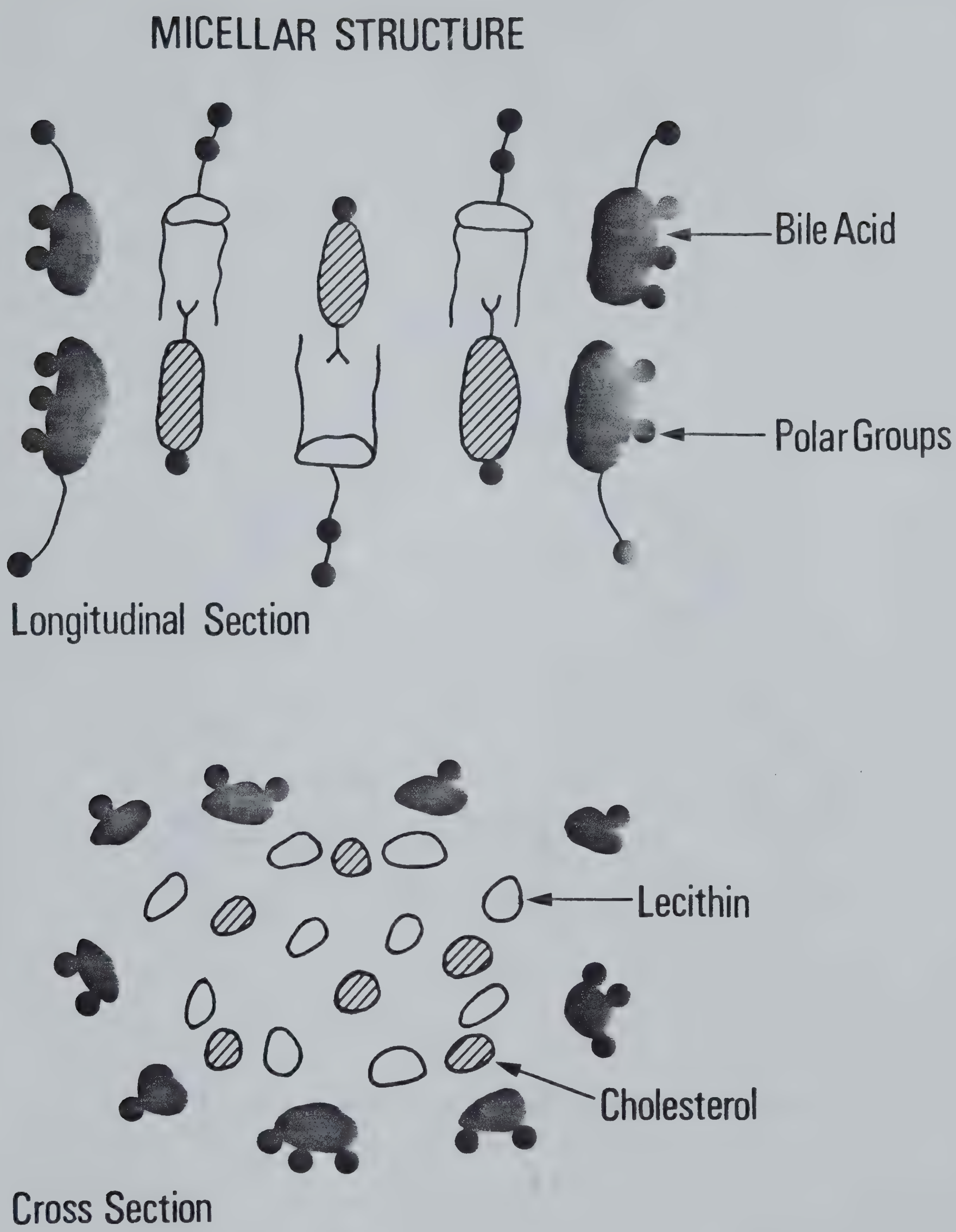
Spanner, in 1932, hypothesized that cholesterol precipitation from bile in the gallbladder results from the liver secreting a paucity of bile acids and phospholipids.[54] Early investigations indicated that lecithin was a minor constituent of bile, but later experiments showed that lecithin was present in relatively high concentrations. This information prompted investigations into the possibility of a bile acid-lecithin system of cholesterol solubility.[55] By the early 1950's Isaksson showed bile from gallstone patients to have a lower ratio of bile acids plus lecithin to cholesterol, when compared to the bile from patients without gallstones.[56] Furthermore, bile acid-lecithin

mixtures were found *in vitro* to have great dissolving powers for cholesterol in aqueous solutions. This verified that lecithin, in combination with bile acids, is indispensable for solubilizing cholesterol in bile.[57]

Detailed investigations of the bile acid-lecithin-cholesterol-water system were undertaken by Small in the 1960's. Initially the ternary system, lecithin-bile acid-water, was studied using X-ray diffraction and microscopic techniques on mixtures with varying concentrations of these substances. Information about the physical state of bile, its behaviour and organization into liquid crystals and micelles on the basis of changing concentrations was extrapolated from these experiments.[58] Similarly, a second ternary system, lecithin-cholesterol-water was investigated. Cholesterol can be incorporated into the structure of a liquid crystal aqueous lamellar phase formed by lecithin and water. Total incorporation will occur provided that the cholesterol-lecithin ratio is less than one to one. Lecithin molecules form bimolecular sheets (liquid crystals) in water with their polar groups oriented to water. The paraffinic portions of the molecules are oriented side to side. Cholesterol molecules can be inserted in the hydrophobic portion of the structure and thus achieve solubility.[59] The quaternary system of bile acids-lecithin-cholesterol-water was investigated by the same methods and the limits for cholesterol solubility defined. It was discovered that the resultant structure is able to solubilize a great deal more cholesterol than the bile acids or lecithin alone.[60 61]

Through these investigations bile acids were found to act like detergents for cholesterol. Beyond a critical concentration in water (critical micellar concentration or CMC) they form polymolecular aggregates termed micelles. This process is also temperature dependent and below a critical temperature (Krafft point) micellar formation will not occur. Such aggregation can only occur with molecules termed amphipaths: that is, possessing appropriately arranged hydrophobic and hydrophylic regions. The structure of a micelle is such that hydrophylic portions are hidden in the center of the aggregate. Micelles can solubilize non-polar molecules such as cholesterol by "dissolving" them in their hydrocarbon center. Mixed micelles (micelles formed from more than one component) are created through the aggregation of bile acids and lecithin in water, such that a coin-like disc is formed. (Fig. 1) A bilayer of bile acids form the edge, while a double layer of lecithin molecules forms the top and bottom. Dissolved cholesterol is interdigitated among the lecithin molecules.[62] [63] [64] [65] A pre-requisite for gallstone formation was hypothesized as oversaturation of bile with cholesterol (i.e., cholesterol concentration above the capacity of micelles for solubilization).[60] [61]

A triangular phase diagram for relative percentage concentrations of cholesterol, phospholipids and bile acids was eventually derived and a line of maximum cholesterol solubility in bile as a function of bile acid and phospholipid concentrations was calculated. (Fig. 2). A collection of bile analyses from patients with and without gallstones was gathered from the literature. When their



MICELLAR STRUCTURE
Bile Acid
Polar Groups
Longitudinal Section
Lecithin
Cholesterol
Cross Section

Figure 1

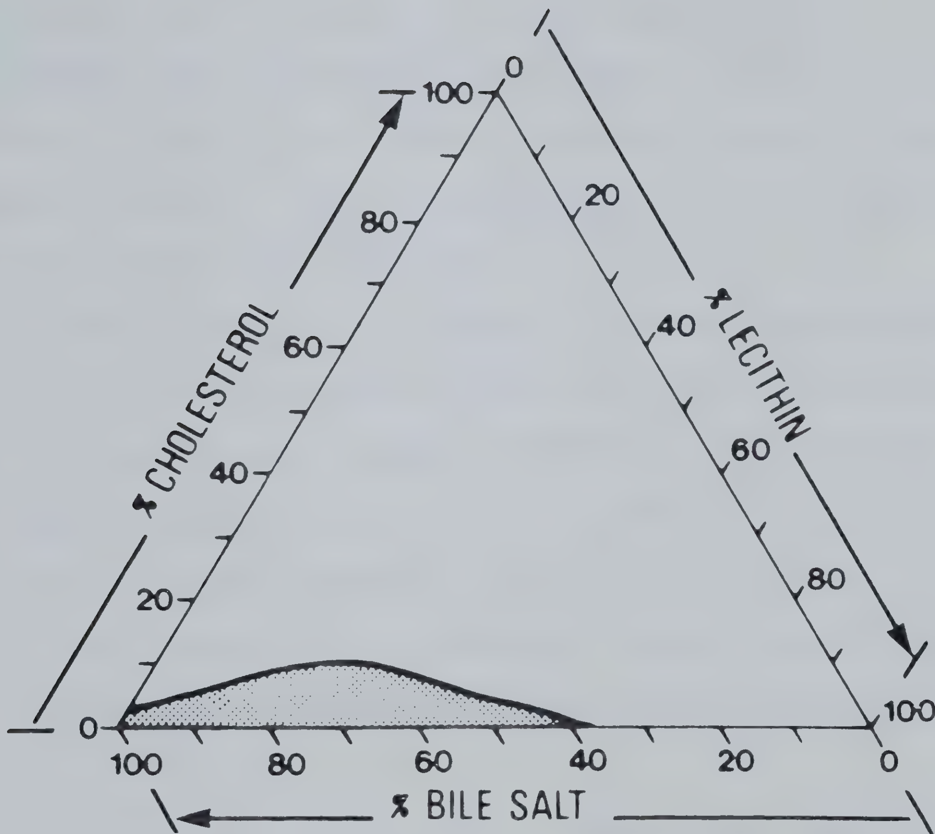


Figure 2. Triangular Co-ordinates of Admirand and Small

bile composition was plotted on triangular co-ordinates a separation of normal from lithogenic bile was obtained on the basis of cholesterol saturation.[66] In 1968, Admirand and Small produced a series of bile analyses obtained from normal and gallstone-containing gallbladders. When the results were plotted on triangular co-ordinates, pathological human bile was supersaturated with cholesterol when compared to normals.[5] Thus the physicochemical basis for cholesterol gallstone formation was established.

Many investigators in numerous countries have since confirmed that bile from patients with cholesterol gallstones is supersaturated with cholesterol. The geographic incidence of gallstones has in part been accounted for since low risk populations have a lower incidence of supersaturated bile than high risk populations. As added proof, animals which do not spontaneously form gallstones, such as the dog, pig, hamster, and Rhesus monkey, have markedly unsaturated bile.[3,7,67,68,69,70]

A few investigators, such as Holzbach, have recently disputed Small's line of maximum cholesterol solubility, and proposed new lines which indicate a lesser ability of bile acids and lecithin to solubilize cholesterol.[71,72,73] It would appear that the area representing the difference between these lines is a zone of supersaturation in a form of delayed equilibrium, termed metastability. Supersaturated bile found in control subjects by these investigators almost implies that their bile may contain inhibitors of nucleation.[74] Most investigators, however, adhere to the original line of cholesterol

solubility and find it provides good clinical separation of gallstone bile from "normal" bile (which the other solubility lines do not).

As suggested above, some studies fail to separate normal from gallstone bile.[75 76] There are many possibilities to account for the observed discrepancies. The time of sampling is important, since it has been shown that fasting bile is more lithogenic than bile collected postprandially.[77] The source of bile also invalidates comparison of some studies since hepatic bile is more lithogenic than gallbladder bile.[78 79 80] As pointed out in an editorial by Sutor, the variety of methods of handling the bile specimen (such as freezing versus leaving it at room temperature) and the numerous available analyses for bile acids, lecithin and cholesterol, may be the reason for discrepancies among some studies.[81] Furthermore, many investigations are carried out in populations at high risk for the development of gallstones. It is probable that many so-called normals in such populations will be in a pre-stone stage of the disease, and have lithogenic bile.[82]

Through experiments analyzing hepatic bile the origin of lithogenic bile was found to be the liver and not the gallbladder. In fact, it has been proposed that cholelithiasis may be a liver disease.[83] The burning question remains, namely "Why does bile become lithogenic?" Hypotheses attempting to answer this question require a review of investigations concerning bile acid, cholesterol, and phospholipid metabolism and the control of biliary secretion for their understanding.

BILE ACID METABOLISM AND PHYSIOLOGY

INTRODUCTION

Bile acids are naturally occurring detergents synthesized in the liver from cholesterol and secreted into bile. One of their important properties is to form micelles which allows them to solubilize biliary cholesterol. Cholesterol, being a lipid, would otherwise be insoluble in bile, since its composition is about 95% water. After reaching the intestines via the biliary tract, bile acids have an important function in lipid digestion and absorption, mainly due to their detergent like action as micelles.

The bile acids, as does cholesterol, undergo an enterohepatic circulation. (Fig. 3). Other endogenous substances such as bile pigments, cholesterol, vitamin B_{12}, folic acid and estrogenic sterols, and exogenous substances such as digitalis glycosides and some antibiotics, also have an enterohepatic circulation.[84] Bile acids have been termed primary and secondary. The primary bile acids are synthesized in the liver, secreted in bile, intermittently stored in the gallbladder between meals, and finally enter the duodenum where they act as a digestive juice.[84] Resorption into the portal system occurs along the length of the small bowel, with maximum absorption in the distal ileum. A small portion of the bile acid pool reaches the colon where bacterial enzymes alter the chemistry of the primary bile acids, producing the so-called secondary bile acids, some of which are absorbed and enter the portal venous system along with the primary bile acids.[85] The intestinal absorption of bile acids is

The Enterohepatic Circulation of Bile Acids

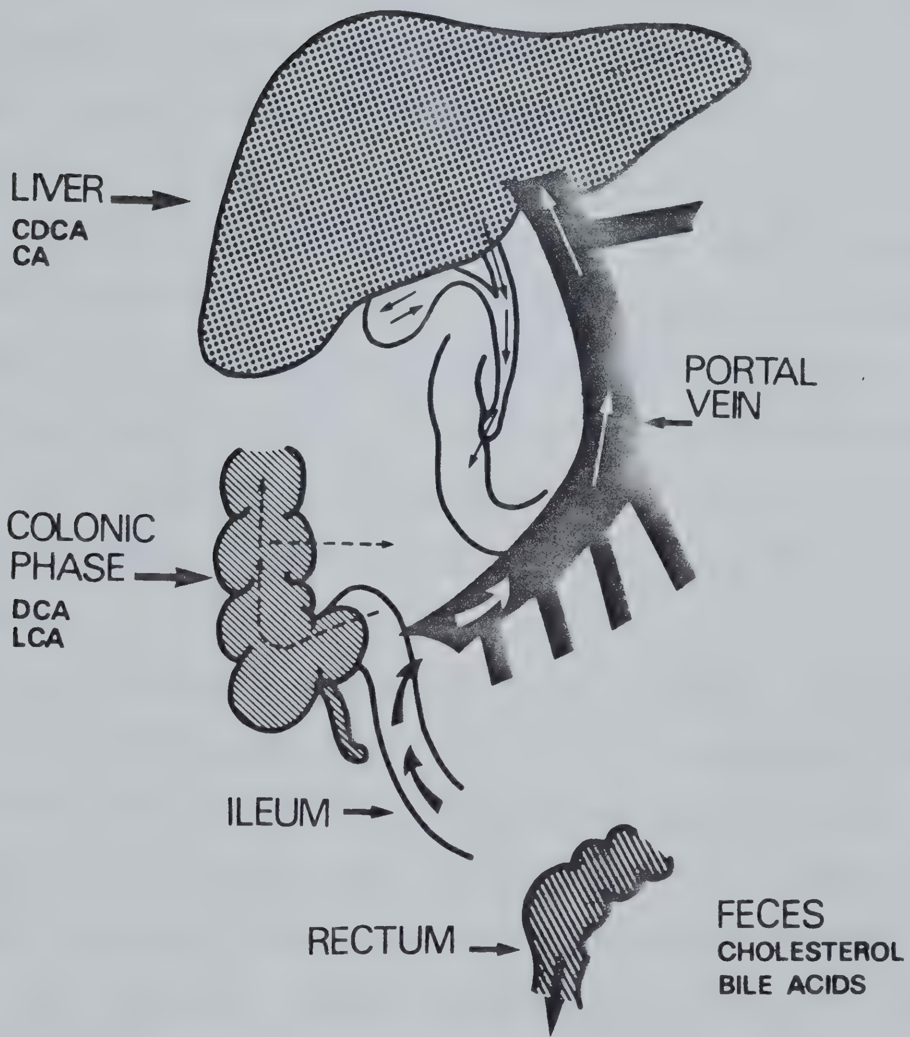


Figure 3

extremely efficient, only 2 - 5% of the total daily circulating bile acids (or 20 - 25% of the total bile acid pool) escape resorption.[84] After the portal blood flow reaches the liver, the bile acids are extracted by the hepatocytes and are resecreted into bile, thus completing the enterohepatic circulation. The only loss of bile acids (the main metabolites of cholesterol) from the system is in the feces.[84]

Since bile acids are essential for the solubilization of biliary cholesterol, and are also the main products of cholesterol catabolism, an understanding of bile acid metabolism, physiology of the enterohepatic circulation, and of surgical and pharmacological alterations to the above, will help to clarify the state of knowledge about cholesterol gallstones and perhaps atherosclerosis.

STRUCTURE

The naturally occurring bile acids in higher vertebrates are derivatives of 5β-cholanoic acid (Fig. 4), a 24-carbon-atom steroid possessing the characteristic cyclopentanophenanthrene nucleus. 27-carbon bile acids are found in lower vertebrates.[86] There are two isomeric cholanoic acids: one in which the plane of fusion of rings A/B is cis-orientated, whereas in the other it is trans (the allo bile acids). It is the cis-form which occurs in man.[86] Data from several studies indicate the following structure for the common bile acid: A/B ring cis, B/C and D/C rings trans. With reference to the methyl group at C_{10} the carbon atoms at C_5 and C_8 are cis, and those at C_9 and C_{14} are trans, and the methyl group at C_{13} is cis. Hydroxyl

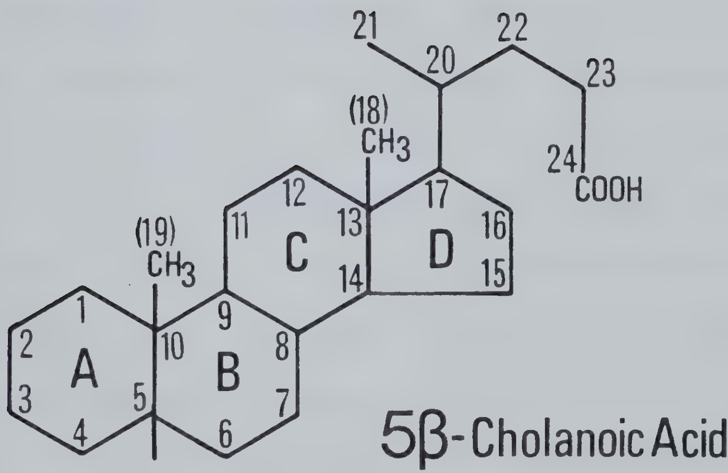


Figure 4. Structure of Cholanoic Acid

groups are in the α-configuration.[87 88] The amino acids, glycine or taurine, are conjugated to the C_{24} carbon.[89 90]

The result of this steric arrangement is to yield a kinked molecule such that the hydroxyl groups, which can be added at the 3, 7 and 12 positions, the amide carbonyl of the peptide bond, and charged carboxylate or sulfonate group (in conjugated bile acids) are orientated to one side of the molecule. Thus the bile acids are molecules with a hydrophilic and a hydrophobic side allowing them to form micelles in an aqueous solution, and to solubilize lipids.[91]

Bile acids are synthesized from cholesterol by the hepatocyte, these are termed the primary bile acids. There are two 24-carbon primary bile acids in man: chenodeoxycholic (CDCA) and cholic acid (CA). In addition, two 27-carbon bile acids are produced in trace amounts: trihydroxycoprostanic acid and dihydroxycoprostanic acid.[92] Upon reaching the colon the primary bile acids are attacked by bacterial enzymes which transform them into the so-called secondary bile acids. Lithocholic (LCA) and deoxycholic (DCA) acids are the major secondary bile acids in bile, although numerous others are present in feces.[84]

SYNTHESIS

The initial step in bile acid synthesis (Fig. 5) is hydroxylation of cholesterol at the 7 position by 7α-hydroxylase to produce 5-cholestene-3β, 7α-diol.[93] The reaction occurs in the microsomal fraction of liver homogenates and requires both NADPH and molecular oxygen. The electron transport system involving cytochrome P-450

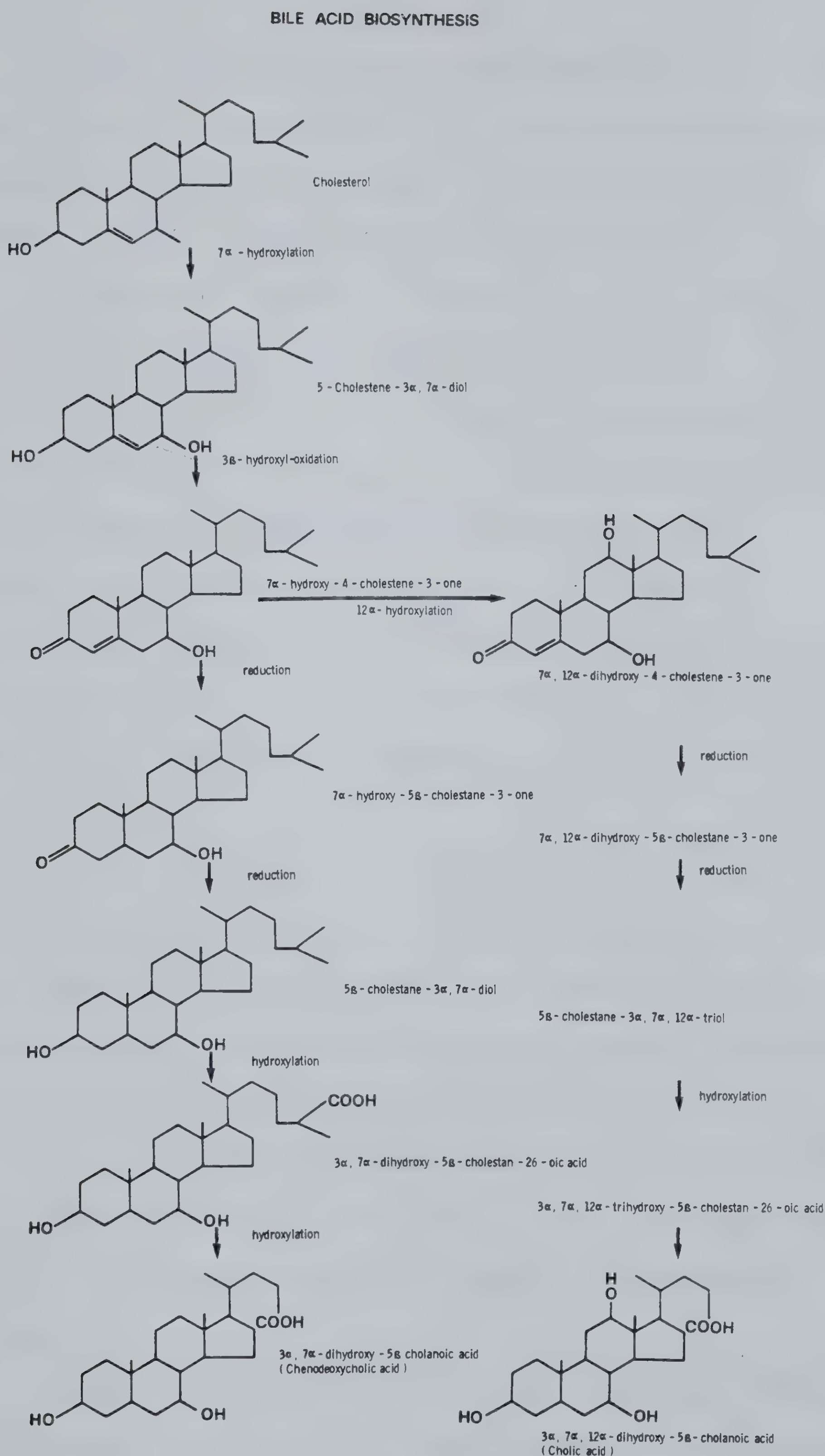

BILE ACID BIOSYNTHESIS
Cholesterol
HO
7α - hydroxylation
5 - Cholestene - 3α, 7α - diol
OH
3β - hydroxyl-oxidation
7α - hydroxy - 4 - cholestene - 3 - one
12α - hydroxylation
7α, 12α - dihydroxy - 4 - cholestene - 3 - one
reduction
7α - hydroxy - 5β - cholestane - 3 - one
7α, 12α - dihydroxy - 5β - cholestane - 3 - one
5β - cholestane - 3α, 7α - diol
5β - cholestane - 3α, 7α, 12α - triol
hydroxylation
COOH
3α, 7α - dihydroxy - 5β - cholestan - 26 - oic acid
3α, 7α, 12α - trihydroxy - 5β - cholestan - 26 - oic acid
3α, 7α - dihydroxy - 5β cholanoic acid
(Chenodeoxycholic acid)
3α, 7α, 12α - dihydroxy - 5β - cholanoic acid
(Cholic acid)

Figure 5

seems to be the rate limiting factor for 7α-hydroxylation.[94] 5-cholestene-3β-7α-diol is then converted to 7α-hydroxy-4-cholestene-3-one by oxidation of the 3β-hydroxyl group, a step catalyzed by the microsomal fraction and NAD.[95] Next, CA and CDCA synthesis diverge with 12α-hydroxylation leading to the formation of CA, whereas reduction of the 5-double bond ultimately produces CDCA.

The 12α-hydroxylation of 7α-hydroxy-4-cholestene-3-one, during the formation of CA, produces 7α, 12α-dihydroxy-4-cholestene-3-one, again dependent on NADPH and the microsomal fraction.[96] 7α-12α-dihydroxy-4-cholestene-3-one is reduced to 5β-cholestane-3α, 7α, 12α-triol through an intermediate 7α, 12α-dihydroxy-5β-cholestane-3-one.[97] Mitrochondrial enzymes then oxidize the side chain through a series of steps involving coenzyme A to produce 3α, 7α, 12α-trihydroxy-5β-cholanoic acid (CA).[98]

Conversion of cholesterol to CDCA follows the same basic structural changes that occur in CA synthesis except that no 12α-hydroxyl group is introduced. (Fig. 5). The sequence from 7α-hydroxy-4-cholestene-3-one is 7α-hydroxy-5β-cholestane-3-one to 5β-cholestane-3α, 7α-diol to 3α, 7α-dihydroxy-5β-cholestanic acid. Sequential hydroxylation of the side chain then proceeds as with CA to produce 3α, 7α-dihydroxy-5β-cholanoic acid (CDCA).[92] Thus, the trihydroxy bile acid CA, and the dihydroxy bile acid CDCA, are synthesized from cholesterol.

Prior to secretion into the biliary system the bile acids are conjugated to either glycine or taurine (amino acids) in approximately a 3:1 ratio, although dietary availability of taurine

can easily change this ratio.[99] Conjugation is important for maintaining solubility in the acid conditions of the upper small intestine during digestion. Free bile acids are insoluble below a pH of 6.[100] Conjugation produces a molecule with a more acidic carboxyl group and the neighbouring amide group induces an electron shift which causes the pK_a to be lower than that of the free bile acid.[101, 102, 103] Absorption of the bile acids is also influenced by conjugation as will be discussed.

Synthesis of conjugated bile acids involves forming a peptide bond between the carboxyl group of the bile acid and the amine group of the amino acids, glycine or taurine. An activating mitochondrial enzyme requiring Mg^{++} or Mn^{++} plus ATP attaches the bile acid (CA) to coenzyme A. This complex reacts with glycine or taurine catalyzed by cholyl taurine (or glycine) acyl transferase to yield glycocholic (or taurocholic) acid plus coenzyme A. A similar reaction occurs with CDCA and with unconjugated secondary bile acids reaching the liver.[104]

The secondary bile acids are produced from primary bile acids by the enzymatic action of intestinal (colonic) bacteria. Hydrolysis of conjugated to free bile acids is carried out by a high proportion of strains of anaerobic bacteria, Bacteroides, Eubacterium, Clostridium, Veillonella, and also Enterococci such as Streptococcus faecalis.[105] Dehydrogenases active at the 3, 7, and 12 positions have been characterized and isolated, 7α-dehydrogenase being by far the most common dehydrogenase among the various bacteria.[106] Resultant products of this

activity are the keto bile acids. This reaction is essential for allowing further dehydrogenation of the steroid nucleus. The responsible enzymes are also produced by a wide variety of bacteria, including E. coli. Dehydroxylation is another common reaction. Only the enzyme for removing the 7α-hydroxyl group has been demonstrated, it is produced by many strains of anaerobic bacteria and Streptococcus faecalis.[107] 7α-dehydroxylation produces the two common secondary bile acids found in bile: 3α-monohydroxy-5β-cholanoic acid (LCA) from CDCA, and 3α, 12α-dihydroxy-5β-cholanoic acid (DCA) from CA.[108]

Other enzymes are capable of nuclear dehydrogenation and aromatization of the steroid rings. Clostridia produce 3-oxo-cholanoyl Δ^4-dehydrogenase and 3-oxo-cholanoylΔ^1-dehydrogenase which can react with keto bile acids to introduce a C_{1-2} and a C_{4-5} double bond.[109] Sequential aromatization of the A, B and C rings by other bacterial enzymes can result in the formation of polycyclic aromatic steroids.[105] With the exception of DCA and LCA there is no significant absorption of fecal (secondary) bile acids. These two bile acids enter the portal system, are removed by the hepatocyte conjugated to taurine or glycine, and secreted into bile. The following bile acids are commonly isolated from feces: from CA, 12α-hydroxy-3-keto-5β-cholanoic acid, 3β, 12α-dihydroxy-5β-cholanoic and 3β-hydroxy-12-keto-5β-cholanoic acid; from CDCA, 3β-hydroxy-5β-cholanoic acid (isolithocholic) and 3-keto-5β-cholanoic acid.[92 110 111] Many other fecal bile acids have been isolated in trace amounts.

Deconjugation, dehydrogenation, dehydroxylation, and

aromatization alter the physicochemical properties of the bile acids, and will be discussed further in following sections.

THE ENTEROHEPATIC CIRCULATION

Bile acids undergo an enterohepatic circulation. (Fig. 3). After secretion by the liver and intermittent storage in the gall-bladder overnight and between meals they enter the intestines. Absorption into the portal venous system occurs along the length of the bowel, but principally in the distal ileum. Secondary bile acids are produced from primary bile acids and absorbed in the colon. After travelling through the portal system they are taken up by the hepatocytes, reconjugated if necessary, and re-excreted, thus completing an enterohepatic circulation.[84 108]

The circulating bile acids comprise a pool which has been estimated at 2 to 4 gms.[112] Size of this pool can be determined by isotope dilution following the intravenous or oral administration of a radiolabelled bile acid and its recovery in bile, as pioneered by Lindstedt.[113] It has been estimated that this pool circulates six to ten times per day, resulting in a total daily bile acid secretion averaging about 30 gms per day.[113 114] Daily (24-hour) secretion in a given individual has been shown to be relatively constant. In other words, pool size times recycling frequency is constant. Individuals with small pools have high recycling rates, large pools recycle less frequently.[115 116]

As mentioned, the only significant loss of bile acids from the enterohepatic circulation is in the feces. Normally 200 to 600 mg

are lost daily.[84] Bile acid synthesis occurs in response to this loss so that the 24 hour secretion (pool times cycles) can remain constant. Without compensatory synthesis the pool would eventually be lost. Synthesis of bile acids is apparently under a negative feedback control at the level of the enzyme 7α-hydroxylase.[117 118 119] Insufficient return of bile acids to the liver stimulates synthesis whereas a sufficient return will turn off 7α-hydroxylase; that is, the reaction is concentration dependent. Acute interruption of the circulation, as in surgical drainage of the common bile duct, causes synthesis to reach a maximum after several hours, approaching four to five times normal in the primate.[84] CA synthesis can increase to 2.1 gm per day, CDCA to 1.2 gm per day, for a total of 3.3 gm per day. Under these conditions the normal CA to CDCA ratio changes from 1.2 to 3.2.[120] There is a critical and somewhat narrow range of return below which no inhibition occurs, and above which no synthesis occurs.[121] It is likely that inhibitory levels are similar for all hepatocytes. However, since hepatocytes take up and secrete bile acids from the portal radicles, there will be a concentration gradient from the portal system to the central vein in any liver lobule. Only when there is sufficient bile acid present to overwhelm the absorptive capacity of hepatocytes near the portal system will the hepatocytes bordering the central vein be inhibited.[108] Factors which increase or decrease bile acid return to the liver (fecal excretion, absorption) will therefore have a reciprocal effect on bile acid synthesis.

Secretion

The circulation of bile acids begins with secretion by the hepatocyte into the bile canaliculi. The most widely accepted theory regarding the formation of bile places primary importance on the secretion of organic ions, of which bile acids are quantitatively the most important.[122] It is felt that the osmotic effect provided by bile acids causes water, electrolytes, and other components of bile to enter the canaliculi.[123] Active transport of bile acids is presumed to be the mechanism for their secretion. Three lines of evidence support this concept:

1. The high ratio of hepatic bile/plasma concentrations of bile acids.
2. The saturation kinetics of secretion.
3. Evidence for competition in the secretion of different anions.[122]

Following collection of canalicular bile by the hepatic ducts, bile enters the common bile duct. Bile can continue to flow down this duct to the duodenum, or it can travel through the cystic duct to be stored in the gallbladder between meals. Gallbladder contraction in response to cholecystokinin-pancreozymin (CCK-PZ) released by food, makes the stored bile acid pool available to the intestines for lipid digestion.[124] A significant portion of bile escapes gallbladder storage so that fasting duodenal bile frequently contains bile acids.[125] Gallbladder function adds a diurnal variation to the secretion and synthesis of bile acids with the most pronounced effect occurring during the overnight fast imposed by sleep. During sleep much of

the bile acid pool is stored in the gallbladder, thus bile acid return to the liver will be at its lowest and therefore synthesis will be increased.[77] Because of the low return to the liver, 7α-hydroxylase is released from inhibition and synthesis of bile acids will reach its 24 hour maximum.

The gallbladder mucosa increases bile acid concentration by the active transport of sodium chloride from bile followed by the osmotic extraction of water.[126] The mucosal barrier is impervious to bile acids and other organic compounds and they become concentrated.[127] It has been shown that deconjugated bile acids can be absorbed rapidly if the pH of gallbladder bile favours the un-ionized or salt state. Similarly, mucosal injury, as in inflammation, promotes the absorption of bile acids.[26]

The gallbladder's effect on enterohepatic cycling can also influence the relative proportions of primary and secondary bile acids. Storage prevents exposure of the bile acid pool to colonic bacteria. If gallbladder function is lost due to surgical removal, impaired due to the presence of calculi, or if contraction stimulation is reduced by protein and fat restricted diets, DCA will increase relative to the proportion of primary bile acids.[128 129 130]

Absorption

Once in the duodenum bile acids function in lipid absorption. However, in order to prevent loss in the feces the bile acids are absorbed along the length of the gut. Absorption is affected by structure of the bile acid, conjugation, and pH. The mechanisms of

intestinal transport have been summarized by Dowling[84] and are:

1. Active transport from the ileum alone, which is the major route for bile acid absorption.
2. Passive absorption from stomach, jejunum, ileum and colon, which may be either passive monomer diffusion or passive micellar diffusion.

Active bile acid transport occurs only in the distal ileum and is quantitatively the main site of absorption. This process has been well investigated in the past few years using the inverted gut sac technique of Wilson and Weisman.[131] The technique is to incubate an inverted sac of gut with physiological saline containing bile acids, this solution is also added to the sac. Transport is assessed by measuring concentration differences inside and outside the sac. High mucosal serosal concentration ratios demonstrate active transport. The process is energy dependent and also requires the presence of extracellular sodium ions; a sodium dependent "pump" has been postulated.[132] Dihydroxy bile acids are less efficiently absorbed than trihydroxy bile acids, and taurine conjugates are more rapidly absorbed than glycine conjugates.[133] There is competitive inhibition of glycine and taurine absorption, suggesting the bile acids share a common transport mechanism.[134]

Passive absorption (diffusion) of bile acids, although quantitatively much less than active absorption, is nonetheless important. Physical properties are the determinants of passive absorption. Diffusion occurs by two mechanisms, passive ionic and

passive non-ionic diffusion. In the first case, movement is driven by an electro-chemical gradient, in the second case, the solute diffuses as the undissociated acid through the lipid barrier of the mucosal cell membranes, concentration difference being the driving force. Anions of both conjugated and unconjugated bile acids can be absorbed. Quantitatively five to six times more bile acids are absorbed by non-ionic diffusion.[135]

Non-ionic diffusion depends on the bile acid being in the un-ionized state which depends on the pK_a of the bile acid and the intestinal lumen. At normal intestinal pH of 5.0 to 7.0, a high proportion of free bile acids will be in the un-ionized state (pK_a 5.0 - 6.3). More glycine conjugates will be ionized (pK_a 4.3 - 5.2) while taurine conjugates will be almost entirely ionized (pK_a 1.8 - 1.9).[84] In summary, free bile acids are easily absorbed in the duodenum and jejunum, taurine conjugates depend on active ileal absorption and glycine conjugates can be absorbed by active and passive mechanisms.[136] The pattern of absorption is designed to preserve high concentrations of bile acids in the intestinal lumen until fat digestion is complete, following which the bile acids are absorbed in the distal ileum to prevent fecal wastage.

The colon can probably absorb any unconjugated bile acid. The major fecal bile acids are DCA and LCA.[137] Rate of colonic absorption can be sufficient to influence the amount of bile acids synthesized by the liver and certainly determines the proportion of secondary bile acids in bile.[138] The rate of bacterial deconjugation

and dehydroxylation determines the amount of colonic absorption by changing the pK_a's of the bile acids. Thus DCA is partially un-ionized and some precipitates while LCA is totally insoluble and only a small amount is absorbed. The numerous other identified fecal bile acids appear in bile in trace amounts, if at all.[64]

A multitude of factors can influence the metabolism of bile acids by their effects on intestinal absorption. Surgical procedures such as ileectomy produce bile acid wasting;[108] colectomy eliminates secondary bile acids from bile.[139] Diseases such as sprue or any severe diarrhoeal state can produce bile acid wasting.[140] Stagnant loop syndrome and bacterial overgrowth will result in increased deconjugation and dehydroxylation of bile acids, which will affect absorption of bile acids and their relative proportions in bile.[141] Drugs such as cholestyramine, or diets high in plant fiber, promote the excretion of bile acids by binding bile acids, and decreasing intestinal transit time, resulting in decreased absorption, and therefore increased synthesis.[142, 143]

Bile acids reaching the portal vein are bound to albumin and transported to the liver.[144] The bile acids are extracted by the hepatocytes utilizing a very efficient active process, resulting in 95% extraction; that is, only 5% of the circulating bile acids reach the systemic circulation.[145] Systemic or serum bile acids occur in extremely low concentrations, about 2 - 4 μmoles per liter.[146] These levels have been observed to increase three- to fourfold post-prandially, reflecting absorption of gallbladder bile released during digestion.[147] High levels have been observed in obstructive

jaundice, intrahepatic cholestasis and pruritis of liver disease.[148]

Bile acids in the systemic circulation are filtered and then returned to the circulation by active processes in the kidneys, a small amount being excreted in the urine. Normally only about 2% of the total bile acid loss can be accounted for by urinary excretion.[149] Urinary excretion can increase several fold in hepatobiliary disease and cholestasis, with a concomitant increase in the percentage of sulfate bile acids.[150]

Sulfation of bile acids by the liver has been shown to occur at the 3, 7, 12 hydroxyl positions by the formation of sulfate esters. The introduction of sulfate groups alters the metabolism, excretion and toxicity of bile acids.[150] Urinary excretion of bile acids is markedly increased by sulfation in patients with hepatobiliary disease, whereas non-sulfated bile acids are rapidly absorbed in the proximal renal tubule.[151 152] Sulfated bile acids are in addition rapidly eliminated in the feces because they precipitate.[153] Some bile acids, particularly LCA, have hepatotoxic properties.[154] It has been shown that lithocholate sulfation is an important metabolic pathway in man, since it increases fecal and urinary excretion, and also diminishes the hepatotoxic properties of this bile acid.[150] Sulfation of bile acids and urinary excretion becomes significant only in hepatobiliary disease.[151] The bulk of bile acids, are, however, absorbed by the hepatocyte, reconjugated if necessary, secreted into the gastro-intestinal tract and reabsorbed, thus completing the enterohepatic circulation.[84]

Function

Bile acids have several important physiological functions:[155]

1. They are responsible in part for the control of biliary secretion.
2. Bile acids are the main catabolic products of cholesterol, and as such have an important role in cholesterol metabolism.
3. Cholesterol transport in bile is dependent on incorporation into micelles formed by bile acids and phospholipids.
4. Intestinal digestion and absorption of lipids relies heavily on the detergent-like action of bile acids.
5. Bile acids have a tremendous influence on water and electrolyte transport in the intestinal tract.

Bile acids are in part responsible for the biliary secretion of water and solutes.[156] It has been estimated that approximately half the biliary water secretion is bile acid dependent.[157] As has been mentioned, bile acids are responsible for their own secretion; high secretion rates depend on high rates of return to the liver. Cholesterol output is dependent on bile acid secretion, although at low bile acid output there is some bile acid independent secretion of cholesterol.[158] Similarly, phospholipid (lecithin) secretion and synthesis requires the presence of bile acids.[157, 159]

Quantitatively, bile acids are the main catabolic excretory products of cholesterol except for small amounts lost as adrenocortical hormones in the urine and feces. This fact has focussed increasing interest on the role bile acids may play in cholesterol

homeostasis, hypercholesterolemia, and consequently atherosclerosis.[155] Disorders such as hepatobiliary obstruction lead to increases in serum cholesterol and hepatic synthesis.[160] Conversely, if bile acid excretion is augmented by ileal resection or cholestyramine therapy, serum cholesterol has been shown to drop.[161 162]

Cholesterol transport in bile depends on incorporation into mixed micelles formed by bile acids and phospholipids. The pioneering work by Admirand and Small on cholesterol solubility in bile has shown that cholesterol cholelithiasis is due to cholesterol being secreted in excess of micellar capacity.[5] This fact has been substantiated by numerous other investigators.[57 66 163] Although the cause of supersaturated bile has been attributed to the gall-bladder,[67] liver,[79] ileum,[162] colon,[164] and diet,[165] there is no question that cholesterol solubility depends on the presence of bile acids.

Intestinal digestion and absorption of fatty acids, tri-glycerides, monoglycerides, cholesterol, and fat soluble vitamins, require their incorporation into bile acid micelles.[7] The stimulus of a meal releases cholecystokinin-pancreazymin which induces gall-bladder contraction and releases pancreatic enzymes for digestion.[124] Triglyceride droplets are hydrolyzed in the small intestine by pancreatic lipase to produce monoglycerides and fatty acids.[166] Mono-glycerides and fatty acids are incorporated into mixed micelles with bile acids. This is a pre-requisite for their uptake by mucosal cells and probably results from mass transfer by collision of the micelle

with the mucosal cell membrane.[167] Only medium chain triglycerides can be absorbed in the absence of bile acids, also medium chain fatty acids can be transported via the portal vein rather than in lymph, as are the other products of lipid digestion.[166 168] Bile acids also shift the pH optimum of pancreatic lipase from 8.0 - 8.5 to 6.5, the pH of the upper small intestine being 6.0 - 6.5.[169] Bile acids are essential for the absorption of cholesterol. Significant concentrations can only be reached in the aqueous phase in micellar form.[7] Furthermore, bile acids enhance the hydrolysis of cholesterol esters by interacting with cholesterol esterase and protecting it from tryptic digestion.[170] Fat soluble vitamins are thought to depend on bile acids for absorption in a manner similar to that of other lipids.[171] The importance of bile acids in fat digestion and absorption is well illustrated by the malabsorption and steatorrhoea seen in patients with ileal resection, a condition producing severe bile acid wasting.[172]

Although little information exists as to the effects of bile acids on water and electrolyte transport in the small intestine, a great deal of information has accumulated regarding their effects on the colon. The cathartic effects of excess bile acids on the colon in ileal disease are well documented.[173 174] In particular, the dihydroxy bile acids inhibit water, sodium chloride and bicarbonate absorption, and promote potassium excretion.[174 175 176] Increased motility resulting in decreased intestinal transit time, occurs with administration of excessive amounts of bile acids.[177] Feeding the

bile acid sequestering agent, cholestyramine, to patients with ileal disorders will improve diarrhoea by adsorbing bile acids. The importance of bile acids in diarrhoeal states has been well documented.[178 179 180 181] Idiopathic bile acid catharsis has even been described. This is a chronic diarrhoeal state in which there is excessive bile acid loss with no other demonstrable cause for the diarrhoea, and is completely relieved by cholestyramine. It is hypothesized that a relative lack of ileal transport sites for bile acids is responsible for the condition.[182]

It can now be appreciated that bile acids play a central role in cholesterol metabolism and in many areas of gastrointestinal physiology. Knowledge of bile acid metabolism and physiology is therefore a pre-requisite to understanding disorders of cholesterol metabolism and gastrointestinal diseases, such as gallstones, diarrhoea, malabsorption, and perhaps atherosclerosis.

BILE ACIDS IN CHOLESTEROL CHOLELITHIASIS

Due to their important function in solubilizing cholesterol in bile, the bile acids have been extensively investigated in gallstone disease. Measurements of bile acid pools, bile acid composition of bile, synthetic rates, and secretion rates have been compared to patients without gallstones.

Pool size determinations have received much attention since Vlahcevic demonstrated reduced bile acid pools in gallstone patients.[183] Reduced pool size has been confirmed by several other investigators

it has been postulated that a reduction in pool size precedes the formation of lithogenic bile. In other words, cholesterol supersaturation may be the result of an absolute deficiency of bile acids.[184 185 186 187 188]

The relative proportions of the four major biliary bile acids in gallstone bile have also been compared to the composition of "normal" bile. An early study indicated an increase in dihydroxy bile acids (CDCA plus DCA) compared to CA in gallstone patients. The ratio of tri- to dihydroxy bile acids was altered from 1.23/1 to 0.65/1 in the gallstone group.[189] More recent studies have shown that the CDCA pool is reduced in gallstone patients.[183 190] In contrast the secondary bile acid, DCA, comprises a much larger percentage of total pool when compared to controls.[185 188 191]

Synthetic rates of the primary bile acids have been estimated by using the rate of loss of radioactivity from bile in patients who have received an isotopic bile acid to calculate the rate of production of new bile acids.[113] In spite of a reduced total pool size in gallstone patients, synthetic rates are the same as, or only slightly lower than, normal.[183 188]

The measurement of secretion rates of biliary lipids was pioneered by Metzger and Grundy who developed a duodenal perfusion technique.[192] Applying the technique to American Indian women with gallstones was investigated, secretion rates were not dissimilar from controls, suggesting a genetic abnormality may be present in the American Indians.[193] Similarly, Northfield and Hofmann showed

that secretion rates are similar in gallstone and control patients.[194 195] Furthermore, they hypothesized that daily bile acid secretion is a constant, the variables being pool size and recycling rate.[116 195 196] In opposition, Shaffer found reduced bile acid secretion rates in non-obese patients with gallstones.[40] However, conditions were unphysiological in all these studies.

In summary, total pool size is reduced in gallstone patients, percentage CDCA is reduced and DCA is elevated. Synthetic and secretion rates do not appear to be much different from controls.

BILIARY CHOLESTEROL METABOLISM AND PHYSIOLOGY

INTRODUCTION

Cholesterol is an important structural element in the cell membranes of all human tissues. With the exception of the adult brain, all cells are capable of cholesterol synthesis.[197] In the primate under the circumstances of a low cholesterol diet however, up to 97% of synthesis occurs in the liver and small intestine.[198] In the monkey, the liver can account for 80% of synthesis but it appears that hepatic synthesis in man may not be quantitatively similar and only 25% of the serum cholesterol may be synthesized by the liver.[198 199] Exogenous or dietary cholesterol is the other major source of cholesterol in man. Dietary cholesterol accounts for 25 - 40% of the daily supply of new cholesterol and synthesis, estimated at about one to two gm per day, accounts for the remainder.[199 200 201]

Both dietary and endogenous cholesterol are secreted by the liver into bile where the bile acids act as solubilizing agents.

through their formation of mixed micelles with phospholipids. Although a portion of cholesterol undergoes an enterohepatic circulation along with the bile acids, absorption is much less efficient.[84] Cholesterol is lost in the feces along with its major bacterial metabolites, coprostanol and coprostanone, at a rate estimated at between one to three grams per day.[105 202] As discussed, a further loss occurs in the form of bile acids, the main metabolites of cholesterol, in the order of 200 to 600 mg per day.[84] A further loss, estimated at less than 10% of the daily total production, occurs in the urine. This is the result of the loss of the metabolites of adrenal and sex hormones, which were originally synthesized from cholesterol.[203] A further small loss will occur with desquamation. In summary though, the main loss of cholesterol and its metabolites from the body is in the feces.

Following absorption, which is bile acid dependent and occurs mainly in the jejunum, cholesterol enters the lymphatics in the form of lipoproteins.[84 204] Lipoproteins are basically spheres of protein, phospholipids and cholesterol containing triglycerides and cholesterol in their centres. Lipoproteins function in the transport of dietary cholesterol in the blood, just as micelles transport cholesterol in bile. Similarly, endogenous cholesterol enters the systemic pool as lipoproteins.[204]

Cholesterol in the exchangeable pool is excreted by the liver into the duodenum via the bile duct, either as cholesterol, or after 7α-hydroxylation as the bile acids. Cholesterol in the

duodenum mixes with ingested cholesterol and is partially reabsorbed from the jejunum via the lymphatic system, entering the blood circulation in the chylomicra (lipoprotein). The bile acids are efficiently reabsorbed from the distal ileum via the portal vein. Unabsorbed cholesterol and bile acids are excreted in the feces. In so far as other pathways for the removal of cholesterol from the body are negligible, the net flux of cholesterol through the exchangeable pool is equal to the daily excretion of fecal steroids derived from the exchangeable pool. Loss is compensated for by endogenous synthesis and absorption of dietary cholesterol.[205]

STRUCTURE AND SYNTHESIS

Cholesterol is a 27-carbon steroid possessing the four major rings characteristic of the cyclopentanophenanthrene nucleus. (Figs. 4, 5). A, B, and C rings are fused cyclohexane rings in the nonlinear or phenanthrene arrangement and the terminal ring, D, is a cyclopentane ring. There is a β-hydroxyl group on carbon atom three and methyl groups numbered 18 and 19 on carbon atoms 10 and 13 and a double bond at the 5 - 6 position. There is an eight carbon side chain at position 17.[206]

Cholesterol biosynthesis involves the reaction of 18 molecules of acetyl CoA to yield cholesterol plus nine molecules of CO_2. (Fig. 6). Approximately 26 steps are known to occur in the synthesis of this complicated molecule.[207] The initial reactions are common to several metabolic pathways; however, the fourth step, in which beta-hydroxy-beta-methyl glutaryl CoA (HMG-CoA) is converted to

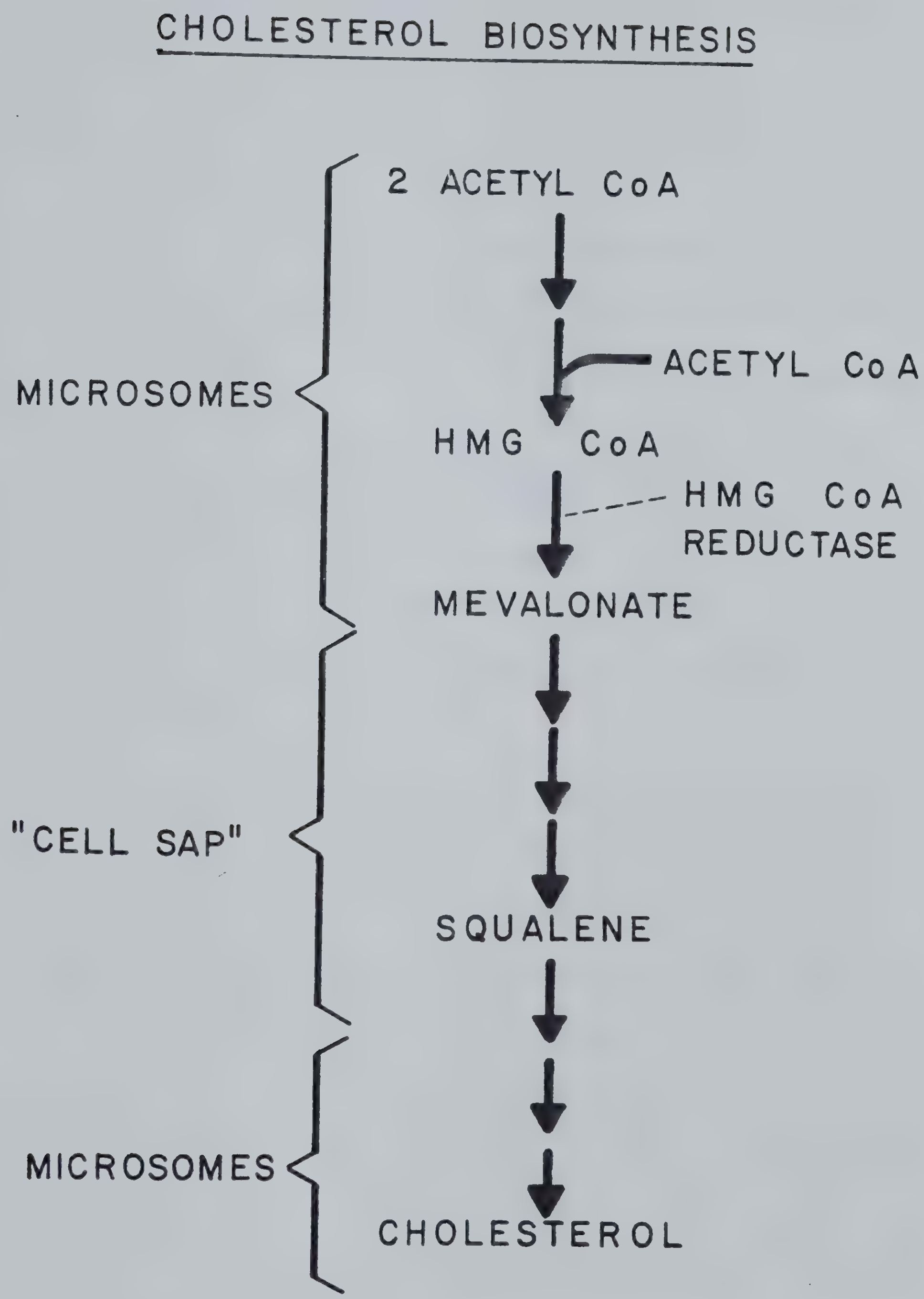

CHOLESTEROL BIOSYNTHESIS
MICROSOMES
2 ACETYL CoA
ACETYL CoA
HMG CoA
HMG CoA
REDUCTASE
MEVALONATE
"CELL SAP"
SQUALENE
MICROSOMES
CHOLESTEROL

Figure 6

mevalonic acid, is a unique and irreversible reaction.[208]

The formation of mevalonic acid from HMG-CoA (formed from three molecules of acetyl CoA) is catalyzed by the enzyme β-hydroxy-β methyl glutaryl CoA reductase in the microsomal fraction of liver cells. This enzyme is the rate controlling enzyme of cholesterol biosynthesis.[203] In the cell sap, six 5-carbon units each derived from mevalonate are condensed to form squalene, a 30-carbon molecule. Again, in the microsomal fraction squalene oxide cyclase catalyzes the anaerobic cyclization of squalene to lanosterol. Conversion of lanosterol to cholesterol involves demythlation of the two angular methyl groups at the 4-position and removal of a third methyl group at the 14-position. The double bond in the side chain and at the B, C ring juncture is saturated and a double bond is introduced at the 5, 6 position.[209 210]

Cholesterol synthesis is under the control of HMG-CoA reductase. Synthesis is inhibited by the presence of cholesterol in a negative feedback fashion.[197] Dietary cholesterol will inhibit liver synthesis, whereas the drug cholestyramine, which interferes with cholesterol absorption will stimulate synthesis.[200 211] Bile acids have been shown to inhibit HMG-CoA reductase activity.[212] A variety of hormones and drugs also influence cholesterol synthesis.[197] Cholesterol feeding does not inhibit intestinal cholesterol synthesis, however, the bile acids do inhibit intestinal synthesis, presumably at the level of HMG-CoA reductase.[213]

Cholesterol is eliminated from the body in the feces mainly

as its major metabolites, the bile acids, or as cholesterol itself. Cholesterol can be attacked by fecal bacteria (Bacteroides, Bifidobacterium, and Clostridia) and can be reduced to coprostanone and coprostanol.[214] Cholesterol, coprostanone and coprastanol are termed the neutral sterols as opposed to the acidic sterols (bile acids). Coprostanol is the main neutral sterol found in the feces of western people and is not absorbed to any significant extent.[105]

TRANSPORT

Cholesterol enters the circulation as lipoproteins which are spherical particles composed of a liquid core of non-polar lipids (triglycerides and cholesterol esters) covered by a mono-molecular film of polar lipids (phospholipids and cholesterol) and apoproteins that differ among the lipoprotein classes.[204] Based on ultracentrifugational and electrophoretic studies there are four major classes of lipoproteins: chylomicrons, very low density (VLDL), low density (LDL) and high density (HDL)lipoproteins.[215] Triglyceride content decreases and cholesterol content increases as density increases.[215]

The apoprotein composition of lipoproteins also varies among the lipoprotein classes. For example, apoprotein B is the major protein of LDL and is present in VLDL. Apoprotein C (really a mixture of C-I, C-II and C-III is a major apoprotein of VLDL and is regularly found in HDL and in minor amounts in LDL. Apoproteins A-I and A-II are the major proteins of HDL. An arginine rich protein has been found in VLDL.[216]

Dietary and endogenous cholesterol enter the lipoproteins and are esterified to long chain fatty acids by plasma LCAT (lecithin cholesterol acyl transferase), an activity highly dependent on Apo A-II. About 75% of the plasma cholesterol is esterified. At body temperatures cholesteryl esters form liquid crystals which are more easily stored and transported than solid crystals of free cholesterol.[217 218] The triglyceride rich chylomicrons are synthesized by the intestinal mucosa. VLDL is synthesized by both liver and intestine.[204] LDL is thought to be a major catabolic product of the metabolism of VLDL and chylomicrons.[217 219] HDL is synthesized in both the liver and intestine.[204]

Cholesterol esters in both chylomicrons and VLDL are rapidly and almost completely taken up by the liver.[204 220] Some exchange occurs, however with HDL during the bi-directional transfer of C-apoproteins with chylomicrons and VLDL.[217 221] Chylomicron and VLDL remnants are converted to LDL through an intermediate density lipoprotein IDL, presumably involving the liver.[204]

The human cell acquires cholesterol necessary for membrane synthesis as LDL.[222] Cellular LDL receptors allow LDL to be taken up intact by the cell.[223 224] LDL is then metabolized to amino acids and free cholesterol which can then participate in membrane synthesis and also suppress endogenous synthesis via HMG-CoA reductase.[225 226]

The liver is the primary site of removal of HDL from the blood.[227] Thus cholesteryl esters in HDL are transported to the organ from which cholesterol is excreted from the body.[204] Evidence is

rapidly accumulating that HDL functions to remove excess tissue cholesterol and return it to the liver.[228 229] Furthermore, HDL remnants have been found in bile.[227]

The exact origin of biliary cholesterol has not been documented. The relative contribution of exogenous and endogenous cholesterol and the exact role the lipoproteins play in formation of biliary lipids is not known.

BILIARY PHOSPHOLIPID METABOLISM

Biliary phospholipids of which 95-98% are lecithin, have an indispensable role in the solubilization of biliary cholesterol, as has been discussed.[230] Although there does not seem to be any major alteration of lecithin metabolism in gallstone patients, as compared to the important alterations in bile acids and cholesterol, the synthesis, metabolism and secretion of biliary lecithin will briefly be reviewed.

Structure of the phosphatidal cholines, otherwise known as lecithin, is based on the glycerol phosphate molecule, i.e., they are derivatives of L-α-phosphatidic acid. The phosphoric acid is bound to glycerol in ester linkage at the terminal α-hydroxyl group. In lecithin the phosphoric acid is bound in ester linkage to choline.[231] The phosphoryl choline group $-O(PO_3)CH_2N(CH_3)_3$ is a zwitterionic hydrophilic group that orientates itself towards the water interface. Two long chain fatty acids (palmitic and linoleic) are esterified to the α and βcarbons of the glycerol molecule, giving the molecule a

hydrophobic side.[232] This structure allows the formation of liquid crystals with water and micelles with bile acids, and the transport of cholesterol in bile.[57 58 59 60 61]

Biliary lecithin is synthesized in the liver, predominantly with preformed choline, via the cytidine diphosphate (CDP)-choline pathway.[233] Choline plus ATP react to form phosphoryl choline. Cytidine triphosphate reacts wtih phosphorylcholine to form cytadine diphosphate choline by the elimination of pyrophosphate. Interaction of cytidine diphosphate choline with 1, 2-diglyceride yields lecithin and cytidine monophosphate.[234]

Secretion and synthesis of biliary phospholipids are both apparently under the control of bile acid secretion.[159] It has been shown during experiments in which the EHC is interrupted that:

1. Bile acid feeding produces a rapidly increased secretion of Phospholipids.
2. Interruption of the EHC of bile acids produces a rapid fall in the incorporation of radiolabelled palmitic acid into lecithin.[159 230 235 236]

Interestingly, phospholipid feeding has no influence on lecithin secretion during interruption of the EHC.[230] The explanation for this observation is that lecithin is rapidly attacked in the intestine by pancreatic lipases which remove fatty acids from the lecithin molecule to produce lysolecithin which is further metabolized.[237] The amount of lecithin which finds its way back to the liver is quite insignificant.[238] Choline may be preserved, however, and there is

evidence that dietary choline has the capacity to influence lecithin synthesis and secretion.[239]

BILIARY SECRETION AND ITS CONTROL

The formation of bile is a very complex and not well understood process. Biliary lipid, water and electrolyte secretion are the most important elements with respect to cholesterol gallstone formation. The bile acids play a central role in the determination of biliary lipid composition and also in water and electrolyte flow.[240] Other compounds which appear in bile such as bilirubin, drugs and vitamins will not be discussed.

Bile acid secretion depends on bile acid return to the liver via the EHC and also bile acid synthesis, which is under negative feedback control at the level of 7α-hydroxylase.[108 117 118 119] As discussed in the section on Enterohepatic Circulation, any factor interfering with this circulation, such as surgical alterations to the gastrointestinal or biliary tract, diarrhoea, or even cholestyramine will interfere with both bile acid re-excretion and synthesis by the liver. The appearance of gallstones in patients with ileal resection illustrates the importance of an intact and properly functioning EHC of bile acids to biliary lipid secretion.[162]

Cholesterol secretion is dependent on the bile acids. The feedback control of hepatic cholesterol biosynthesis by HMG-CoA reductase, the rate controlling enzyme, is influenced by the EHC of bile acids and the absorption of dietary and endogenous cholesterol (a

process dependent on the bile acids' ability to form micelles).[160] 7α-hydroxylase, under bile acid feedback control, whether stimulated or suppressed, will influence the availability of cholesterol for secretion into bile by its rate control over the conversion of cholesterol into the bile acids.[241] In fact, Strasberg has shown, by partial interruption of the EHC in Rhesus monkeys, that cholesterol secretion declines as bile acid synthesis increases.[158 242]

To complicate matters, the different bile acids have different effects on the synthesis of cholesterol and bile acids. For example, CDCA suppresses liver HMG-CoA reductase, whereas the other bile acids do not.[243] DCA feeding has been claimed by Einarsson to reduce CA, but not CDCA formation, whereas Low-Beer showed that DCA has the reverse effect.[244 245]

Phospholipid synthesis and secretion has been demonstrated to be dependent on and increased by, bile acids by several investigators.[157 159 235 246] Phospholipid secretion is not influenced differently by dihydroxy or trihydroxy bile acids.[247]

Another way in which the bile acids may influence cholesterol and phospholipid secretion into bile is by the formation of micelles. It has been suggested that the formation of micelles may be the major factor determining the entry of biliary lipids into bile.[248 249] In both these studies infusion of a non-micellar forming bile acid, dehydrocholate, did not stimulate the flow of biliary lipids in bile fistula rats and dogs. Infusion of taurocholate, which forms micelles, produced a marked increase in biliary lipid flow.[248 249]

A certain portion of cholesterol secretion is not related to bile acid secretion. Below a certain rate of bile acid flow cholesterol secretion is uncoupled.[250 251] This has also been observed by other investigators.[240 243] A bile acid independent phospholipid secretion is apparent from some studies.[230 240]

Bile acid secretion is also thought to be important in the production of canalicular water and electrolyte flow.[159] This chloretic potency is presumed to be due to their osmotic activity.[251 252] A bile acid independent canalicular flow has been observed in the dog, rat, rabbit and man.[157 240 253 254] It is felt that active sodium transport is the mechanism by which this flow occurs.[254 255] Bile salt independent secretion is estimated at 60% in the rat and rabbit.[254 255]

The gastrointestinal polypeptide hormones cholecystokinin, glucagon, gastrin, and secretin also affect biliary secretion. Cholecystokinin has a dual effect. Bile flow is stimulated by cholecystokinin. Resultant gallbladder contraction releases stored bile acids into the EHC, producing increased bile acid secretion, which in turn stimulates cholesterol, phospholipid, water and electrolyte flow.[124] Furthermore, cholecystokinin stimulates the liver to produce a watery bile rich in bicarbonate.[257] Similarly, gastrin, glucagon and secretin stimulate the flow of water and bicarbonate.[258 259 260]

In summary, biliary lipid, water and electrolyte secretion is in part dependent on bile acid synthesis and secretion. A bile acid independent flow has, however, been observed in all mammals

investigated, including man. This independent flow is determined by active hepatocyte and canalicular sodium transport as well as the stimulation of certain gastrointestinal hormones. The importance of knowledge of the mechanisms of biliary lipid secretion in cholesterol cholelithiasis, in which cholesterol secretion may be increased, has been alluded to in the studies of Grundy, Northfield, Shaffer and Strasberg.[39 40 116 158 194]

TREATMENT OF GALLSTONES

Approximately 50% of gallstones found at autopsy were previously unrecognized and likely asymptomatic. Gallstones are diagnosed because of biliary colic, cholecystitis, cholangitis or complications of these in about 30% of patients. Another 15% are diagnosed during the investigation of dyspepsia. The remaining 5% are discovered during an investigation, or abdominal surgery performed for other reasons.[6] Symptomatic gallstones will result in recurrent attacks in 50% of patients within five to ten years.[261] Less than 25% of patients with asymptomatic stones will develop symptoms within ten years.[262]

Because of the danger of gallstones becoming symptomatic or complicated, their removal is recommended. Currently, the accepted method of treatment is cholecystectomy, or in poor risk patients with complications, cholelithotomy.[4] Since the late 1960's attention has focussed on the possibility of dissolving gallstones, a potentially simpler and less costly form of treatment.

In 1937 Rewbridge reported the successful dissolution of gallstones. Working on the postulate that a bile acid deficiency may be responsible for cholesterol precipitation, a crude preparation of bile acids was administered orally to five gallstone patients. Radiographic disappearance of gallstones was recorded in two of these patients following nine months of treatment.[263] Cole reported the disappearance of retained stones from post-operative cholangiograms of seven patients fed bile extracts in 1957.[264] Swell and Bell reported improvement in biliary lipid composition in two of three patients fed bile acids in 1968.[265]

By 1969 Schoenfield and Thistle reported on the effects of feeding pure individual bile acids to gallstone patients.[266] Further experimentation revealed that chenodeoxycholic acid (CDCA) is capable of reducing cholesterol saturation of bile, whereas cholic acid (CA) and deoxycholic acid (DCA) do not have this effect.[190 267] It was also shown that oral CDCA could reduce the lithogenic potential of bile in young Indian women.[82] The successful dissolution of gallstones by CDCA was simultaneously announced by Danzinger and Bell in 1972.[268][269] Danzinger observed the disappearance of stones in four out of seven patients.[268] It was subsequently shown that CDCA dissolved stones, whereas there was no response in patients receiving CA or placebo.[267 269] Since this time world experience in roughly 5,000 patients has confirmed that CDCA is an efficacious agent for gallstone dissolution in the majority of patients.[270] Unfortunately, bile returns to its lithogenic state when therapy is stopped and gallstone

recurrence has been noted.[267 271 272] Although the spontaneous disappearance of gallstones has been observed, the rate is so low (less than 1% per year) that the above results cannot possibly be explained on this basis.[273 274]

The optimum dosage of CDCA has been more or less established at 12-15 mg/Kg, and efficacy at that dose is 50 to 70%.[270 275] Duration of therapy to achieve dissolution in most series ranges from six to 22 months.[243 276] CDCA therapy should be used only in relatively asymptomatic patients who have radiolucent stones (cholesterol, no calcium) in radiologically functioning gallbladders.[276] CDCA therapy is not without side effects. Most patients complain of diarrhoea initially, due to the effects of CDCA on colonic water and electrolyte secretion.[267 268 269 277] Elevations in SGOT occur transiently in about 25% of patients.[270] It is postulated that this rise is due to an increase in the potentially hepatotoxic bile acid, lithocholic acid (LCA), which is formed from the 7α-dehydroxylation of CDCA in the colon.[276] Hypercholesterolemia is a potential complication by blocking of endogenous bile acid synthesis from cholesterol.[278] Careful investigation has not revealed any increase in serum cholesterol, however.[279 280]

The mechanism by which oral CDCA reduces the lithogenicity of bile appears to be the suppression of hepatic cholesterol synthesis.[278] Although it was originally felt that CDCA worked by expanding the bile acid pool, which is usually reduced in gallstone patients, studies using CA to expand the pool have shown this is not

the case.[183 184 268] Furthermore, lithogenicity can be lessened without an increase in pool size with the use of CDCA therapy.[281 282] Biliary lipid secretion studies have shown that cholesterol secretion is decreased during CDCA therapy, while bile acid and phospholipid secretion remain unchanged.[281 283] It is felt that CDCA specifically inhibits HMG-CoA reductase which controls cholesterol synthesis.[270] Reduction of HMG-CoA reductase has been observed in patients treated with CDCA.[284 285]

The 7β-epimer of CDCA, ursodeoxycholic acid, has been shown by Makino in daily doses of 450 mg to 2 gm to dissolve gallstones.[286] A normal constituent in small amounts in human bile, ursodeoxycholate was originally discovered in the bear.[287] Ursodeoxycholate (UDCA) is formed by the action of colonic bacteria on CDCA through the intermediate 7-ketolithocholic acid.[288] Cholesterol content of bile is reduced by UDCA through the suppression of HMG-CoA reductase.[289 290] UDCA may be more valuable in gallstone dissolution than CDCA, since it is not metabolized to lithocholic acid (LCA) in significant amounts.[290] Furthermore, UDCA is apparently devoid of colonic secretory properties and may eliminate the problem of diarrhoea seen in CDCA therapy.[291]

Since low phospholipid concentration has been reported in gallstone patients, and is an important component of micellar structure, phospholipid feeding has been attempted.[292] It is unlikely that this form of treatment will have any success since the absorption of intact lecithin and its EHC is insignificant.[238] Phenobarbital has

been investigated on the basis of its ability to induce liver enzymes and its potential to increase bile acid synthesis by stimulating 7α-hydroxylase. Results however have not been encouraging.[276] Furthermore, phenobarbital increases HMG-CoA reductase activity which would predispose to biliary cholesterol saturation.[285]

In summary, the current treatment of cholelithiasis is surgical. Only in patients with radiolucent stones in functioning gallbladders who are relatively asymptomatic, especially if they are poor surgical risks, should gallstone dissolution be considered.

OBJECTIVES

The objectives of these investigations are:

1. To determine if the colonic phase of the enterohepatic circulation is altered in gallstone patients.
2. To investigate the effects of a high fiber (bran) diet on cholesterol saturation, bile acid composition and pool size in people with and without gallstones.
3. To investigate the effects of a bran diet on cholesterol transport (lipoprotein distribution) in man.
4. To determine the effects of a bran supplement on fecal volume and steroid elimination from the human body.
5. To formulate a hypothesis as to the etiology of cholesterol gallstones and suggest a means for their prevention, based on the observed biochemical and physiological effects of a dietary bran supplement on biliary lipid metabolism.

MATERIAL AND METHODS

PART I: BILE ACID FRACTIONATION

Patients in this study were selected from the operating schedule of the University of Alberta Hospital. Eight patients undergoing cholecystectomy for radiolucent stones in functioning gallbladders were studied. Similarly, six patients undergoing laparotomy for other upper abdominal conditions (hiatal hernia, duodenal ulcer, stomal ulcer, abdominal pain of unknown origin) were selected as controls. (Table 1). The mean age of the gallstone group was 42.5 years, average weight 75 Kg. Mean age and weight of the control group was 48 years and 64 kg.

Patients were excluded from the study on the basis of disease or resection of the large or small intestine. Elevated bilirubins, persistently elevated liver enzyme levels (SGOT, alkaline phosphatase, LDH) or infected bile on aerobic or anaerobic culture similarly resulted in exclusion. Furthermore, only afebrile patients with normal white blood cell counts were accepted. In the control subjects it was deemed necessary to exclude biliary lithiasis as a cause of symptoms by operative cholecystocholangiography.

Bile samples were obtained by aspirating the gallbladder as fully as possible to ensure representative sampling.[294] Stones were obtained from the gallstone patients for analysis. Bile samples were taken immediately to the laboratory and examined for cholesterol

TABLE 1

Age: Sex: Weight I

Pt. No.	Age (Yrs)	Sex	Wt (Kg)	% Cholesterol in Stone(s)
Gallstone Patients				
1.	57	F	65	95
2.	42	M	87	88
3.	29	F	56	68
4.	34	F	60	66
5.	25	M	75	94
6.	65	F	78	79
7.	55	F	112	81
8.	33	F	65	60
Controls				
1.	30	F	64	
2.	24	F	49	
3.	71	M	86	
4.	39	M	63	
5.	70	M	56	
6.	54	M	66	

crystals under a polarizing microscope. Specimens were then mixed and phospholipids extracted at once or the specimens were stored on ice until it was convenient to extract them (never exceeding one hour). Following extraction the specimens were frozen until analysis could be completed. Total solids were determined by comparing dry weight to wet weight of the specimens. Samples below 3% or above 25% total solids were excluded as recommended by Small.[5] All tests were done in duplicate.

Phospholipids were determined by a colorimetric method for inorganic phosphorus developed by Bragden.[295] (Appendix A). Cholesterol was determined by the method of Abell.[296 297] Basically bile was treated with alcoholic KOH to liberate cholesterol from lipid complexes and to saponify any cholesterol esters. Cholesterol was extracted into petroleum ether and the Liebermann-Burchard reaction was used to produce a green color which was measured spectrophotometrically. (Appendix B). Bile acids were determined by the enzymatic method of Engert and Turner, using 3α-hydroxysteroid dehydrogenase.[298] (Appendix C). Cholesterol content of stones was determined by drying the stone in air and grinding it to a fine powder. Using 0.5 gm of powdered stone, cholesterol was determined as outlined above, and percentage cholesterol was calculated.

Relative percentages of bile acids, phospholipids and cholesterol were calculated from the total of their concentration in millimoles for each specimen. Results were plotted on the triangular co-ordinates of Admirand and Small.[5] (Fig. 2). The lithogenic index

of Metzger was then calculated. Lithogenic index is the ratio of actual cholesterol to the theoretical maximum amount of cholesterol that bile can solubilize at that particular concentration of phospholipids and bile acids. Values greater than 1.0 are supersaturated, values less than 1.0 are unsaturated.[299] (Fig. 7).

Bile acid fractionation was done by gas-liquid chromatography on hydrolyzed samples of bile. (Appendix D). Duplicate bile samples were hydrolyzed with Clostridium welchii acetone powder (containing cholyl glycine hydrolase obtained from Sigma Biochemicals, Inc.) to remove taurine and glycine.[300 301 302] The hydrolysate was then acidified with HCl and the free bile acids extracted into diethyl ether.[300 301] Bile acid methyl esters were prepared using diazomethane.[301]

Trifluoroacetate derivatives of the bile acid methyl esters were prepared by adding trifluoroacetic anhydride.[301] Samples were taken up in acetonitrile and injected into a Perkin-Elmer 990 gas chromatograph using a three foot 1.5% QF-1 column. 7-ketodeoxycholic acid was used as an internal standard.[300] (Appendix D).

Pure standard bile acids LCA, DCA, CDCA, CA, (P-L Biochemicals) and pure glychocholic and taurochenodeoxycholic acid (Calbiochem) were added to specimens in each run to obtain recoveries, and also to quantitate sample areas. 7-ketodeoxycholic acid (Steraloids, Inc.) was added to each sample as an internal standard. Quantitation of the bile acids in each specimen was determined by estimation of the area under each peak obtained from the chromatogram. Recoveries

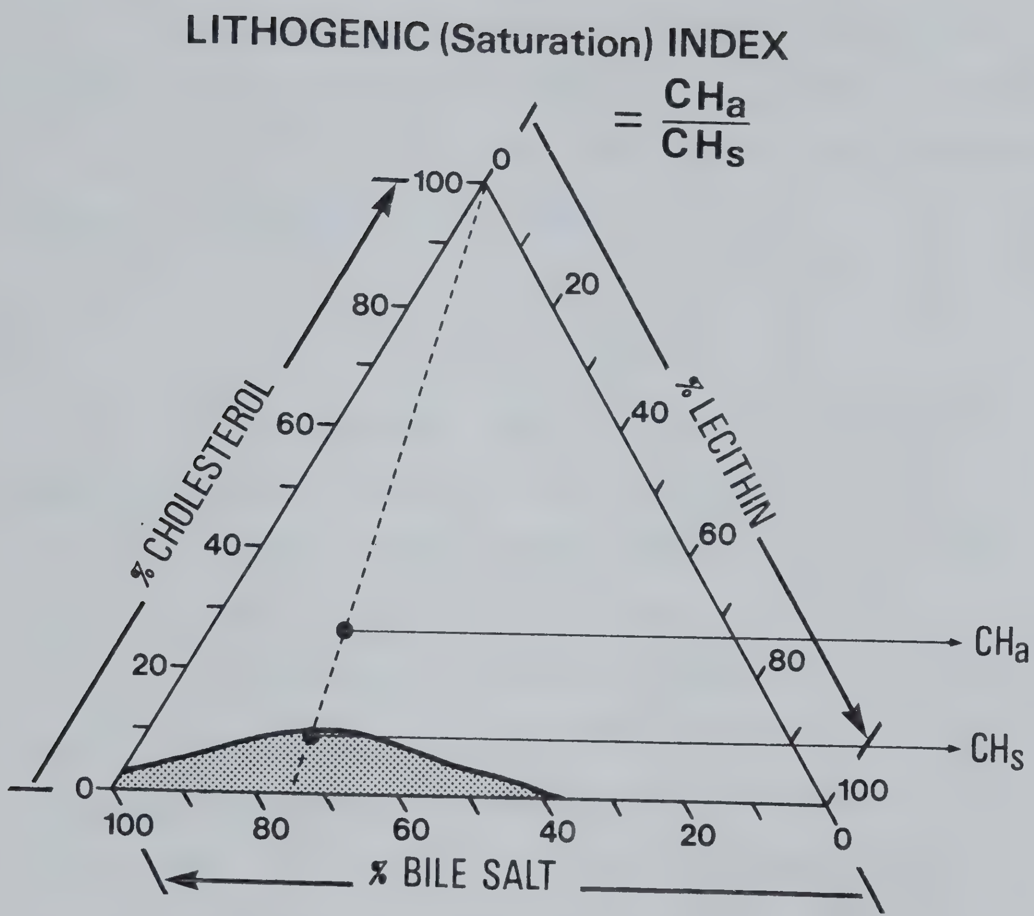


Figure 7

CH_a = Actual cholesterol

CH_s = Theoretical maximum cholesterol

ranged from 91% to 110% and duplicate samples agreed to within 3%.

Statistical evaluation of the data was calculated as in Appendix F, using an Olivetti programma 101 calculator. Students "t" test was used to determine the significance of the difference of mean values between the two groups.

PART II: BRAN DIET

In this study nine control subjects were compared to nine gallstone patients before and after a one month period on a high fiber (bran) diet. Informed written consent was obtained from all volunteers before the experiment was begun.

The nine control patients were selected from a group of the author's acquaintences on the basis of lack of any history of gastro-intestinal pathology and complete lack of any symptoms of biliary tract disease. All controls were judged to be physically fit, healthy, active, diet conscious, co-operative individuals. Mean age of the control group was 23 years, and mean weight was 70 Kg. It was felt that using a young group for controls would lessen the likelihood of these people being in a pre-gallstone state of lithogenic bile.[81] Similarly only two females were included since it is more likely for females to have lithogenic bile than males, based on the sex incidence of gallstone patients. To avoid radiation exposure, oral cholecyst-ograms were not obtained on these volunteers. (Table 2).

The nine gallstone patients were referred to the study and were accepted on the basis of having radiolucent stones in functioning

TABLE 2

Age: Sex: Weight II

Patient	Age	Sex	Weight(Kg)
Controls			
1. Oli	42	M	80
2. Rod	28	M	75
3. Jam	23	M	70
4. Gle	25	M	77
5. Joy	23	F	49
6. Hea	16	F	59
7. Lan	17	M	64
8. Ros	18	M	75
9. Bru	15	M	80
Gallstones			
1. Dav	65	F	68
2. Koz	74	F	80
3. Bel	39	F	100
4. Ing	52	F	68
5. Yak	36	F	75
6. Kat	61	F	57
7. Pat	55	F	70
8. Gni	45	M	82
9. Tam	39	F	68

gallbladders. Only asymptomatic patients who refused surgery for their stones were accepted. Aside from gallstones these patients had no other gastrointestinal pathology and were otherwise healthy with the exception of #1 (Table 2), who had had a sigmoid resection for carcinoma. The mean age of this group was 51 years, and the mean weight was 74 Kg. There was only one male in this group, and since the study was dependent on referral, the male/female ratio was not under our control.

After an initial interview, patients were given an intravenous injection of 4 μCi of ^{14}C-cholic acid in 50% ethanol (New England Nuclear, greater than 98% radiochemical purity by thin-layer chromatography) at approximately 18.30 hours. The injection was given into the tubing of a fast running drip of normal saline and the syringe rinsed several times to ensure complete delivery of the isotope. Following an overnight fast the patient returned to have a single lumen radiopaque enteroclysis tube with a flexible stylet placed in the stomach via the nasogastric route. The tube was then positioned fluoroscopically with the aid of the stylet in the distal descending duodenum.[304 305] Gallbladder contraction was then stimulated by infusing 40 ml of 10% Travasol (Baxter Laboratories), an amino acid mixture, down the tube.[306] The tube was clamped for 10 minutes and then connected to suction. Approximately 10 minutes later, 10 - 15 ml of bile rich duodenal juice was obtained. Elapsed time from isotope injection to collection of the bile sample was 14½ hours ± 30 minutes. Blood specimens were obtained for determination of

total proteins, albumin, alkaline phosphatase, SGOT, LDH, bilirubin, cholesterol, triglycerides, and lipoprotein cholesterol fractionation.

Volunteers were then instructed to add to their daily diet 50 gm (3/4 measuring cup) of Kellog's All Bran. Following one month on the diet, the volunteers returned and the above procedures were repeated.

Bile samples were taken immediately to the laboratory and examined under the polarizing microscope for cholesterol crystals, and phospholipids were extracted into chloroform-methanol at once. The specimens were then frozen until further analyses could be performed.

Biliary lipid analysis was performed as in Part I, with some modifications. Total bile acids, cholesterol, and bile acid fractionation were obtained by gas-liquid chromatography. Pure standard cholesterol (Sigma Biochemicals) was added to the standard mixture of bile acids (LCA, DCA, CDCA, CA) and to bile specimens to obtain recoveries. After preparation (Appendix D) the trifluoroacetate derivatives of the bile acid methyl esters were injected in acetonitrile into a Hewlett Packard 5830A gas chromatograph connected to a Hewlett Packard 18850A computerized terminal and recorder. Using the standard bile acid plus cholesterol mixture, and 7-ketodeoxycholic acid as an internal standard, the computer was programmed to calculate the amount of the individual bile acids and cholesterol in each sample. This was performed by calculating the area of each peak in the chromatogram and comparing them to the peak

areas of a known amount of the standards, and to the area of the internal standard in the standard mixture and the sample to compensate for variations in injection. Recoveries ranged from 90% - 110%, and duplicate samples agreed to within 3%.

Phospholipids were determined by the method of Bragden.[295] (Appendix A). Cholesterol and total bile acids were determined as in the previous study (Appendix B and C) as a double check on the results obtained by gas-liquid chromatography. The lithogenic index (Fig. 7) was then calculated from the molar percentages of bile acids, phospholipids and cholesterol. The relative percentages of the four bile acids was calculated from the amounts calculated by the Hewlett Packard 18850A terminal.

Pool size was determined by isotope dilution 14½ hours after an intravenous injection of ^{14}C-carboxylcholic acid (4 μCi).[306] Radioactivity in the bile sample was determined by liquid scintillation counting, using a Nuclear Chicago Isocap/300 counter, following decolorization of bile, using hydrogen peroxide to eliminate color quenching.[307] Decolorized bile and standard isotope were added to Unogel Emulsifier (Schwarz Mann) and each sample was counted for ten minutes. Pool size was calculated by the following formula after constructing standard graphs and specimen graphs of counts per minute, vs. volume of sample and extrapolating the graph to a one ml volume. (Appendix E):

$$\frac{\text{isotope standard cpm/ml} \times \text{ml isotope injected} \times \text{mg bile acids/ml}}{\text{bile sample cpm/ml} \times 1000} = \text{pool size (gm)}$$

Total protein, albumin, alkaline phosphatase, SGOT, LDH and bilirubin were determined by an SMA 12/60 autoanalyzer under the auspices of the University of Alberta Hospital Department of Laboratory Medicine. Similarly, total serum cholesterol and triglycerides were determined by standard automated enzymatic methods using an Abbott auto-analyzer.

Serum lipoprotein fractionation was determined by the following method. High density lipoprotein (HDL) was separated from low density (LDL) and very low density (VLDL) by precipitation of LDL and VLDL with heparin and manganese chloride, according to Burstein, and the LDL+VLDL precipitate solubilized with sodium citrate.[308] Cholesterol was measured directly in both fractions and in the native serum by the cholesterol-esterase (cholesterol oxidase reaction) using a commercial kit (Boehringer Mannheim cat. no. 15738).[309] The Abbott bichromatic analyzer was used to determine cholesterol following the above procedures.[310] Lipoprotein ratios were calculated by the simple formula:

$$\frac{\text{VLDL+LDL}}{\text{HDL}} = \text{Lipoprotein ratio}$$

The significance of the mean values of results between the two groups was calculated using the Students "t" test. Significance of results before and after bran was calculated using the "paired" Students "t" test. (Appendix F).

PART III: FECAL BILE ACIDS AND NEUTRAL STEROLS

In this study fecal weight, fecal bile acids and neutral sterols were quantitated before and after a 50 gm daily bran dietary

supplement. Four volunteers (Table 3) were obtained from the University of Alberta Hospital and the Glenrose Provincial Hospital. Volunteers were selected on the basis of a chronic physical disability because it seemed necessary that they be longterm in-patients in order to monitor their intake of bran, and to ensure accurate stool collections. All volunteers had a negative history of biliary tract or other intra-abdominal pathology, and all were judged to be healthy and fit, aside from their reason for hospitalization (venous stasis ulcer, and rehabilitation following hip arthroplasty and patellectomy). Only one patient was on medication (#3 - Cloxacillin - Tables 3, 11). Laxatives were discontinued at the onset of the experiment. Informed consent was obtained from all volunteers.

Three females and one male with a mean age of 56 years, and a mean weight of 66 Kg were included in this experiment. All patients except #3 complained of chronic constipation; #4, for example, only had a small bowel movement every to seven days over many years. At the onset of the experiment blood was obtained for determination of total proteins, albumin, alkaline phosphatase, SGOT, LDH and bilirubin to ensure normal liver function. A 72 hour stool collection was obtained, after which the patients and nursing staff were instructed to supplement the daily diet with 50 gm of All Bran. After three to four weeks a second blood sample and 72 hour stool sample were obtained.

Analysis of blood samples were again performed by automated methods, using an SMA 12/60 autoanalyzer. Each weighed stool specimen

TABLE 3

Age: Sex: Weight III

Patient	Age	Sex	Weight(Kg)
1. Ock	40	M	102
2. Han	60	F	51
3. Mor	56	F	59
4. Pal	69	F	52

was homogenized, and a 0.5 gm sample was extracted into ethyl acetate following alkaline hydrolysis. (Appendix G). Total bile acids and neutral sterols were then quantitated by GLC on a Hewlett Packard 5830A gas chromatograph, following derivatization of the extract as in Appendix D, and Bran Diet methods. Daily output of feces, bile acids, neutral sterols (cholesterol and coprostanol) were then calculated, using the weight of the 72 hour stool collection. Statistical significance of results before and after bran was calculated using the "paired" Students "t" test. (Appendix F).

RESULTS

PART I: BILE ACID FRACTIONATION

The gallstone group, which consisted of two males and six females (Table 1), all had lithogenic bile. Table 4 shows the data for biliary lipid composition (cholesterol, bile acids, and lecithin). Mean lithogenic index for this group was 1.4. Cholesterol crystals were observed in the bile of all these patients. The mean cholesterol content of the gallstones removed from these patients was 79%. (Table 1)

The control group consisting of four males and two females (Table 1) all had unsaturated bile. Biliary lipid data is presented in Table 3. Mean lithogenic index for this group was 0.65. Cholesterol crystals were not observed in the bile of these patients. Figure 8 shows the biliary lipid data from both groups plotted on the triangular co-ordinates of Admirand and Small. Clear separation of the gallstone group from the control group is demonstrated on the basis of cholesterol saturation.

The proportions of CA, CDCA, and DCA in gallbladder bile from the eight gallstone patients and the six controls is shown in Figure 9. LCA is not indicated since it formed an insignificant proportion of the total bile acids and did not vary between gallstone and control groups. The proportion of DCA in the bile acid pool was significantly higher in the gallstone group (gallstone mean ±SE = 30% ± 2.5, $p < 0.05$). The proportions of the primary bile acids CA

TABLE 4

Biliary Lipid Composition

Patient No.	Millimoles per Liter			Percent of Total Millimoles			Lithogenic Index
	Cholesterol	Bile Acids	Lecithin	Cholesterol	Bile Acids	Lecithin	
Gallstone Patients							
1.	30.6	105.0	36.9	17	62	21	1.7
2.	35.5	177.0	64.6	13	64	23	1.3
3.	10.9	54.0	18.7	13	65	22	1.3
4.	5.3	31.7	10.5	11	67	22	1.1
5.	5.2	29.0	4.3	14	75	11	1.4
6.	5.1	30.0	12.6	11	63	26	1.1
7.	9.5	52.0	16.5	12	67	21	1.2
8.	21.4	58.0	18.1	21	60	19	2.2
Controls							
1.	2.5	46.8	7.7	5	82	13	0.5
2.	14.3	150.6	16.3	8	83	9	0.8
3.	27.8	210.0	94.3	8	64	28	0.8
4.	6.8	62.0	14.9	8	74	18	0.8
5.	3.0	58.0	12.4	4	79	17	0.4
6.	3.1	34.0	14.7	6	66	28	0.6

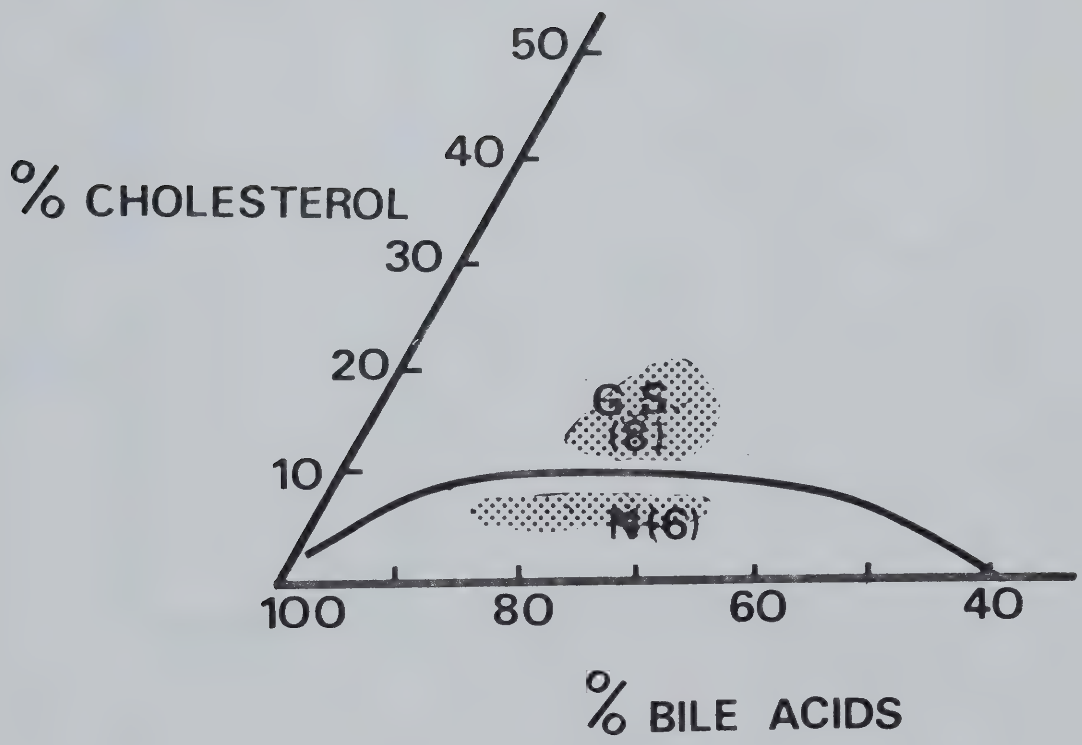


Figure 8. Bile Analyses on Triangular Co-ordinates. Shaded areas represent circles drawn around plotted values for 8 gallstone (GS) patients and 6 patients with normal (N) biliary tracts.

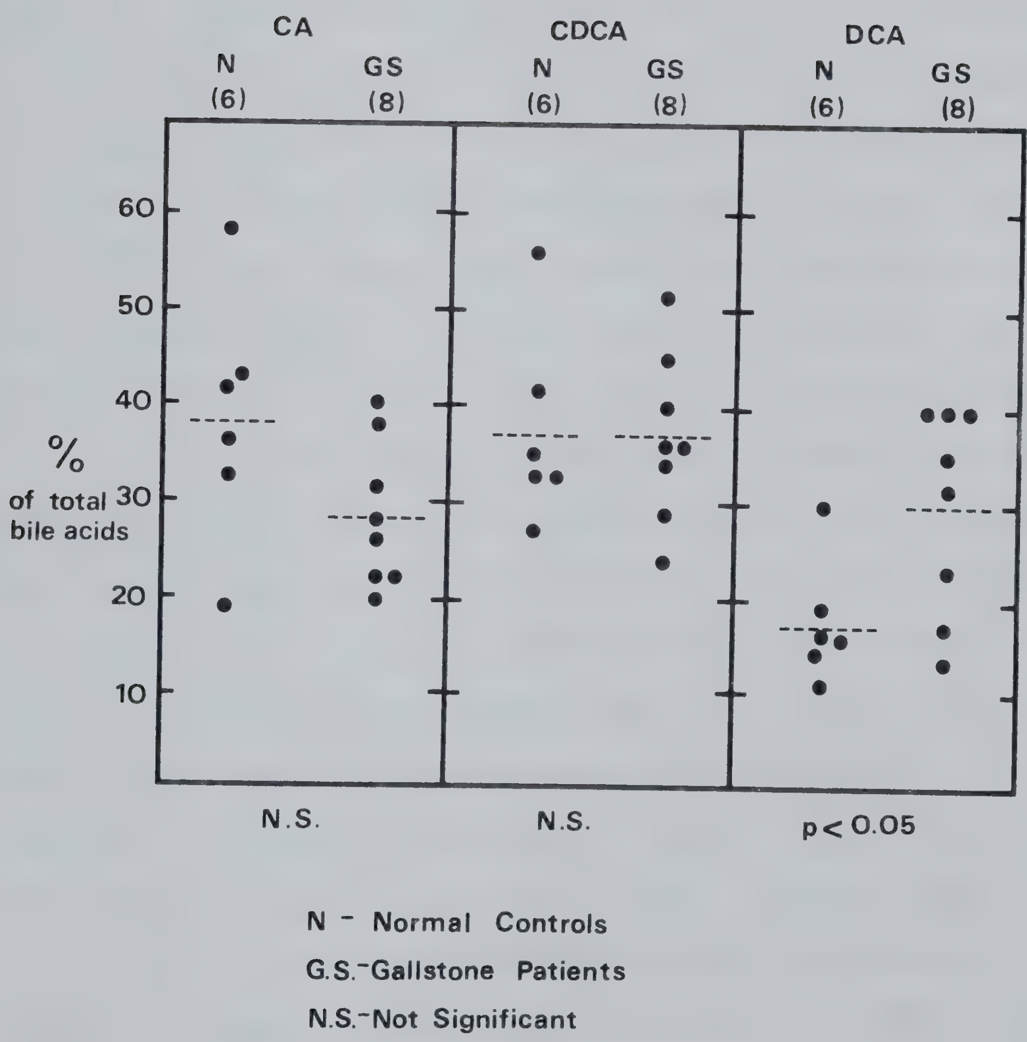


Figure 9. Bile Acid Fractionation

and CDCA were not significantly different.

PART II: BRAN DIET

The control group consisted of seven males and two females. (Table 2). Alkaline phosphatase, SGOT, LDH, total protein, albumin and bilirubin were normal and remained normal throughout the experiment. Cholesterol crystals were not observed in the bile of any of these volunteers. Bile was initially unsaturated (mean lithogenic index ± SE = 0.70 ± 0.1) and demonstrated a trend to drop following the bran diet (lithogenic index = 0.51 ± 0.06 $p < 0.10$), using the "paired" Students "t" test. Complete biliary lipid data are shown in Table 5.

The gallstone group consisted of eight females and one male. (Table 2). Alkaline phosphatase, SGOT, LDH, total protein, albumin and bilirubin were normal and remained normal throughout the experiment. Cholesterol crystals were seen in the bile of several patients before the diet; crystals were not observed in any bile specimens taken after the diet. Bile was initially saturated, or supersaturated, with cholesterol in all gallstone patients. Complete biliary lipid data are presented in Table 6. Initially the mean lithogenic index was 1.43 ± SE 0.16 and became unsaturated in eight of the patients following the bran diet (mean lithogenic index 0.76 ± 0.07, $p < 0.01$). Lithogenic index fell from 2.1 to 1.2 in the ninth. The fall in saturation is shown in Figure 10.

Total bile acid pool size in the control group initially was 2.37 ± 0.36 grams (mean ± SE) and remained essentially unchanged

TABLE 5

Biliary Lipids: Controls

Patient	Bile Acids mM		Bile Acids %		Cholesterol mM		Cholesterol %		Phospholipids mM		Phospholipids %		Lithogenic Index	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
1. Oli	6.45	0.73	61	82	0.9	0.065	9	7	3.1	0.1	30	11	1.0	0.7
2. Rod	24	21	66	78	3.2	1.2	9	4	9.0	4.9	25	18	0.9	0.4
3. Jam	5.5	15.4	63	75	0.8	0.8	9	4	2.3	4.3	28	21	0.9	0.4
4. Gle	26	29.5	76	73	2.2	2.4	6	6	6.0	8.4	18	21	0.6	0.6
5. Joy	19	13.6	80	80	1.1	0.6	5	3	2.7	2.9	15	17	0.5	0.3
6. Hea	57	92	82	81	2.7	6.5	4	6	9.5	14.6	14	13	0.4	0.6
7. Lan	16	4.6	65	68	2.7	0.25	11	4	6.0	1.9	24	28	1.1	0.4
8. Ros	111	11.5	85	69	3.5	0.7	3	4	16.1	4.5	12	27	0.4	0.4
9. Bru	16	17.6	70	71	1.25	1.9	5	8	5.7	4.8	25	20	0.5	0.8
MEAN													0.7	0.51

TABLE 6

Biliary Lipids: Gallstones

Patient	Bile Acids mM		Bile Acids %		Cholesterol mM		Cholesterol %		Phospholipids mM		Phospholipids %		Lithogenic Index	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
1. Dav	3.5	2.02	72	68	0.7	0.25	15	8	0.6	0.7	13	24	1.5	0.8
2. Koz	8.0	16.4	60	67	1.5	2.4	11	10	3.8	5.5	29	23	1.2	0.9
3. Bel	4.2	9.8	67	72	0.72	0.9	11.4	7	1.4	2.9	22	21	1.1	0.7
4. Ing	50	17	65.5	67	9.5	2.6	12.5	10	16.9	5.9	22	23	1.2	0.95
5. Yak	8.5	2.69	56	77	2.5	21.9	17	6	4.1	5.8	27	17	2.4	0.6
6. Kat	17.0	41.2	68	76	3.3	3.6	13	7	4.6	9.4	19	17	1.3	0.7
7. Pat	2.1	10.2	70	67	0.3	0.6	10	4	0.6	4.5	20	29	1.0	0.4
8. Gri	2.3	7.75	71	80	0.35	0.56	11	6	0.57	1.4	18	14	1.1	0.6
9. Tam	1.2	18	67	61	0.4	3.7	22	12	0.2	8.1	11	27	2.1	1.2
MEAN													1.43	0.76

GALLSTONE PATIENTS (9)

LITHOGENIC (SATURATION) INDEX
BEFORE AND AFTER BRAN DIET

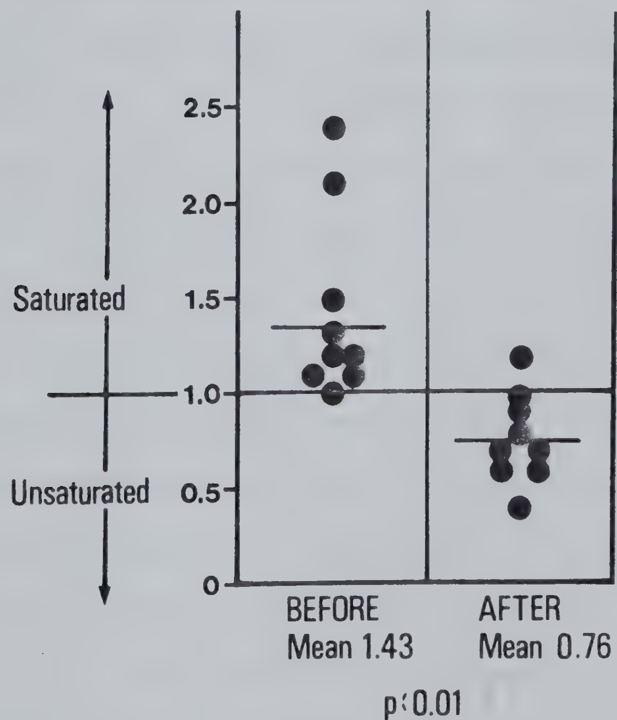


Figure 10

after four weeks on the bran diet, 2.32 ± 0.16 grams (Table 7). Pool size was reduced initially by an average of 0.75 gm ($p < 0.05$) in the gallstone group, when pools were weight corrected to 70 Kg. The uncorrected pools showed a trend to increase in the gallstone group following the bran diet (1.63 ± 0.19 grams vs. 2.03 ± 0.21 grams, $p < 0.10$). Complete data are presented in Table 8 and Figure 11.

Bile acid fractionation showed the proportion of DCA to be increased in the bile of gallstone patients. DCA (Tables 7 and 8, Figure 12) formed 31.3% ± SD 11.21% of the bile acid pool of gallstone patients, compared to 19.0% ± SD 7.53% in the controls ($p < 0.02$). The proportion of DCA in the gallstone group decreased to a mean value of 23.3% ± SE 3.29%, $p < 0.05$ after the bran diet. (Figure 13). CDCA comprised 40.4% ± SD 6.38% of the bile acids in the control group, and was significantly lower in the gallstone group, 28.8% ± SD 8.35%, $p < 0.01$, at the outset of the experiment. (Tables 7 , 8 ; Fig. 14) There was no significant increase in either group after the bran diet. The proportions of CA and CDCA were not significantly different in either group before and after bran. LCA was excluded as in Part I.

Serum cholesterol in the control group was initially 161 ± 9.4 mg% (mean ± SE) and was unchanged after the bran diet at 168 ± 12 mg%. A significant elevation of cholesterol was observed in the gallstone group (219 ± 15.1 mg% $p < 0.01$) and did not change after four weeks on the bran diet (211 ± 17.4 mg%). Complete data are presented in Table 9 and Figure 15. Mean serum triglycerides (Table 9) in the control group was 113 ± 14.7 mg% and did not change with bran

TABLE 7

Bile Acid Pools and Fractionation: Controls

PATIENT	POOLS (gms)		DCA (%)		DC (%)		CDCA (%)	
	Before	After	Before	After	Before	After	Before	After
1. Oli	2.22	2.42	28	33	28	39	44	28
2. Rod	1.22	1.57	26	24	39	38	33.5	38
3. Jam	2.00	3.13	20	18	44	38	35	41
4. Gle	2.54	2.54	25	19	33	42	40	37
5. Joy	1.88	1.86	12	12	56	45	32	42
6. Hea	1.80	2.75	18	12	45	50	36	37
7. Lan	1.28	1.86	26	22	27	41	46	35
8. Ros	3.84	2.07	7	6.5	46	54	45	39.5
9. Bru	4.55	2.65	9	6	39	48	52	46
MEAN	2.37	2.32	19	17	39.7	43.9	40.3	38.2

TABLE 8

Bile Acid Pools and Fractionation: Gallstones

Patient	POOLS (gms) Before	POOLS (gms) After	DCA (%) Before	DCA (%) After	CA (%) Before	CA (%) After	CDCA (%) Before	CDCA (%) After
1. Dav	0.83	1.09	35	20	21	50	38	17
2. Koz	1.79	2.47	23	25	36	28	38	40
3. Bel	1.33	2.22	51	24	32	41	17	34
4. Ing	1.94	1.61	22	28	40	34	37	37
5. Yak	1.90	-	42	29	43	37	15	31
6. Kat	1.74	2.79	39	28	33	45	25	25
7. Pat	1.72	1.37	12	6	47	56	35	38
8. Gni	1.95	2.26	32	27	42	40	26	33
9. Tam	1.73	2.45	26	23	45	43	28	33
MEAN	1.63	2.03	31.3	23.3	37.7	41.6	28.8	32.0

MEAN BILE ACID POOLS BEFORE AND AFTER BRAN DIET

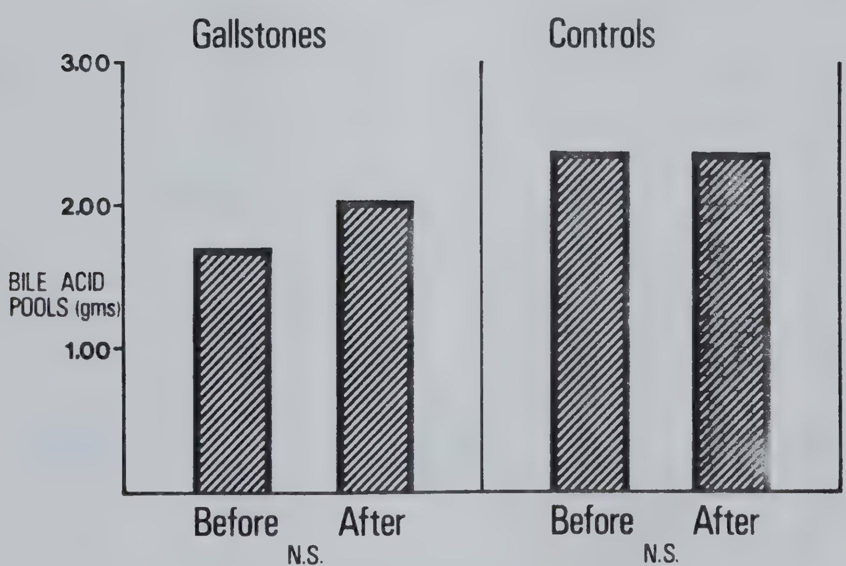


Figure 11

% DCA OF TOTAL BILE ACID POOL
CONTROLS vs GALLSTONES

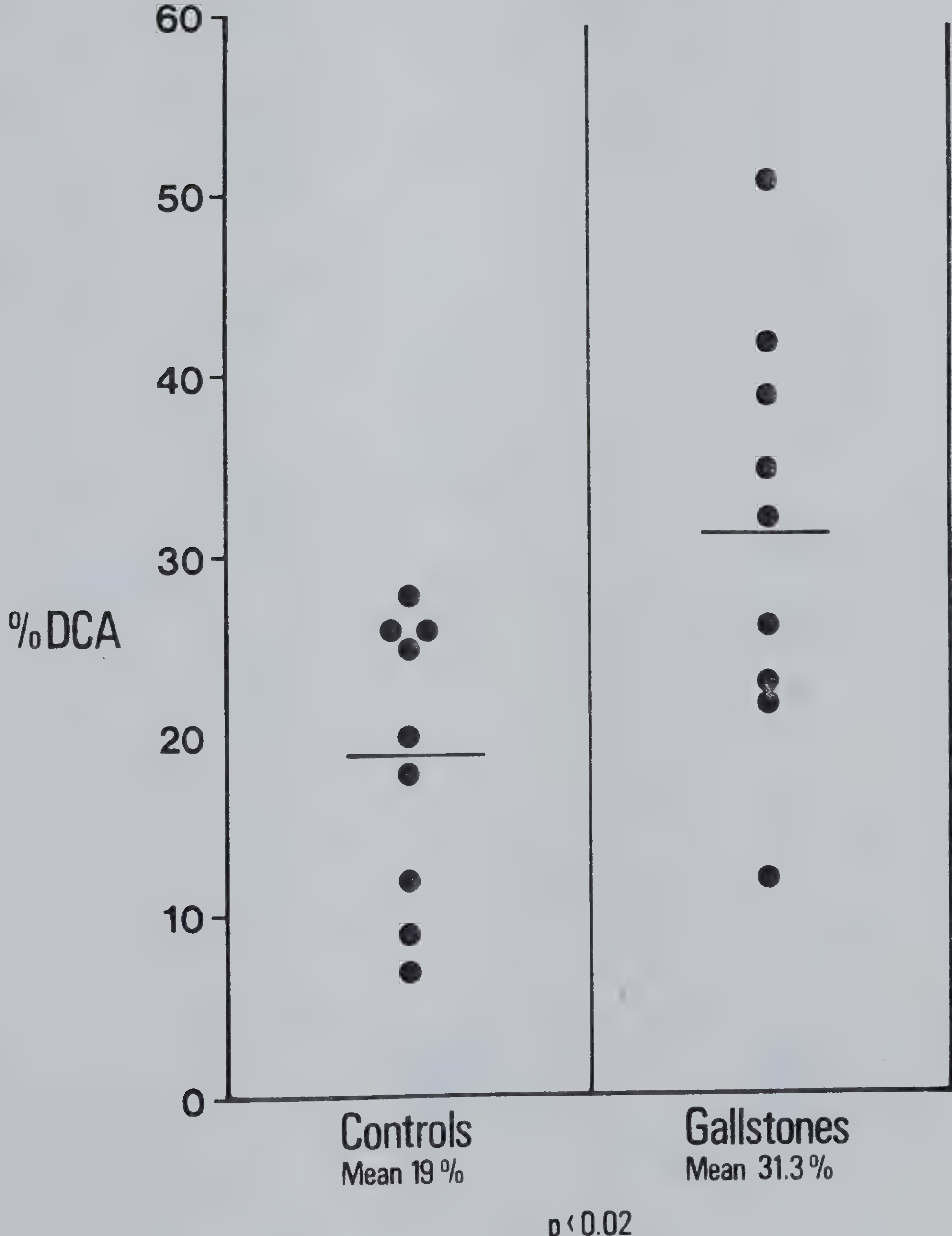


Figure 12

% DCA OF TOTAL BILE ACID POOL IN GALLSTONE PATIENTS BEFORE AND AFTER BRAN DIET

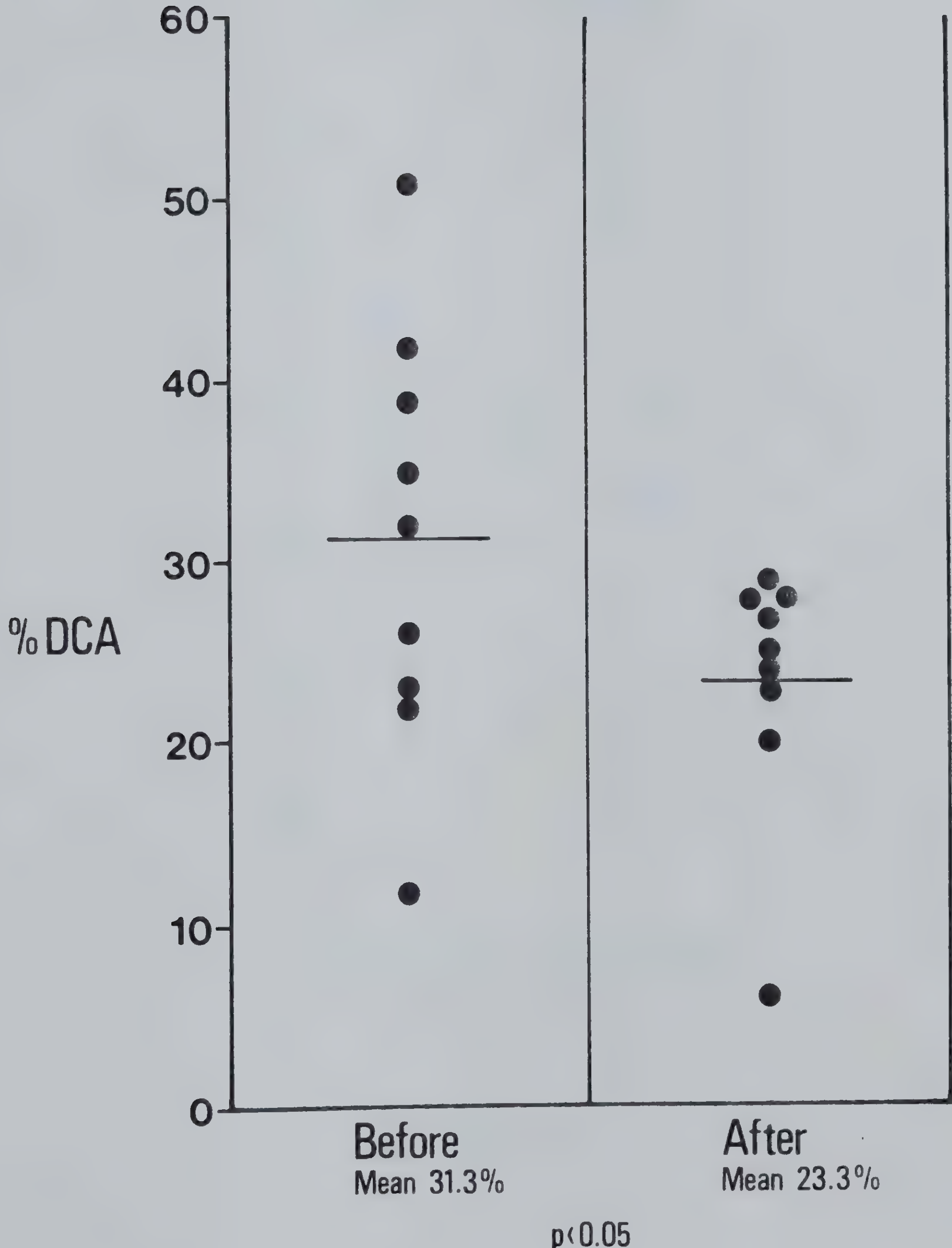


Figure 13

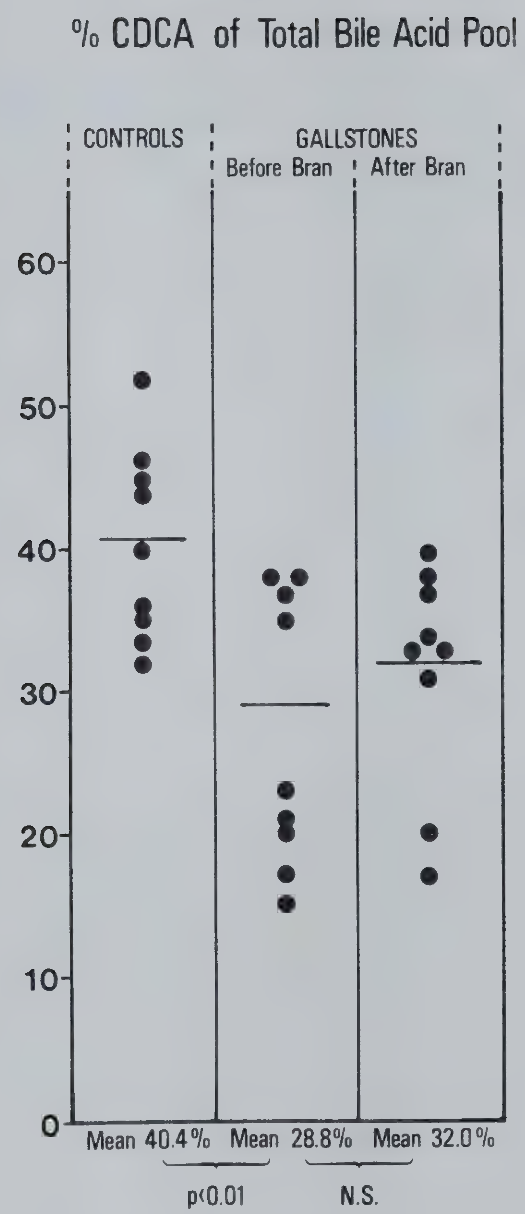

% CDCA of Total Bile Acid Pool
CONTROLS
GALLSTONES
Before Bran
After Bran
60
50
40
30
20
10
0
Mean 40.4%
Mean 28.8%
Mean 32.0%
p<0.01
N.S.

Figure 14

TABLE 9

Total Serum Cholesterol and Triglycerides

PATIENT	CHOLESTEROL (mg%)		TRIGLYCERIDES (mg%)	
	Before	After	Before	After
Controls				
1. Oli	212	227	167	164
2. Rod	165	149	70	151
3. Jam	161	148	68	65
4. Gle	137	144	100	119
5. Joy	147	161	75	73
6. Hea	145	171	112	193
7. Lan	201	231	150	117
8. Ros	152	135	188	53
9. Bru	129	147	95	74
MEAN	161	168	113	112
Gallstones				
1. Dav	279	252	252	114
2. Koz	212	233	212	321
3. Bel	218	130	224	246
4. Ing	269	275	305	368
5. Yak	237	233	116	136
6. Kat	188	218	140	141
7. Pat	201	196	116	149
8. Gni	148	153	194	277
9. Tam	256	266	192	291
MEAN	219	211	195	219

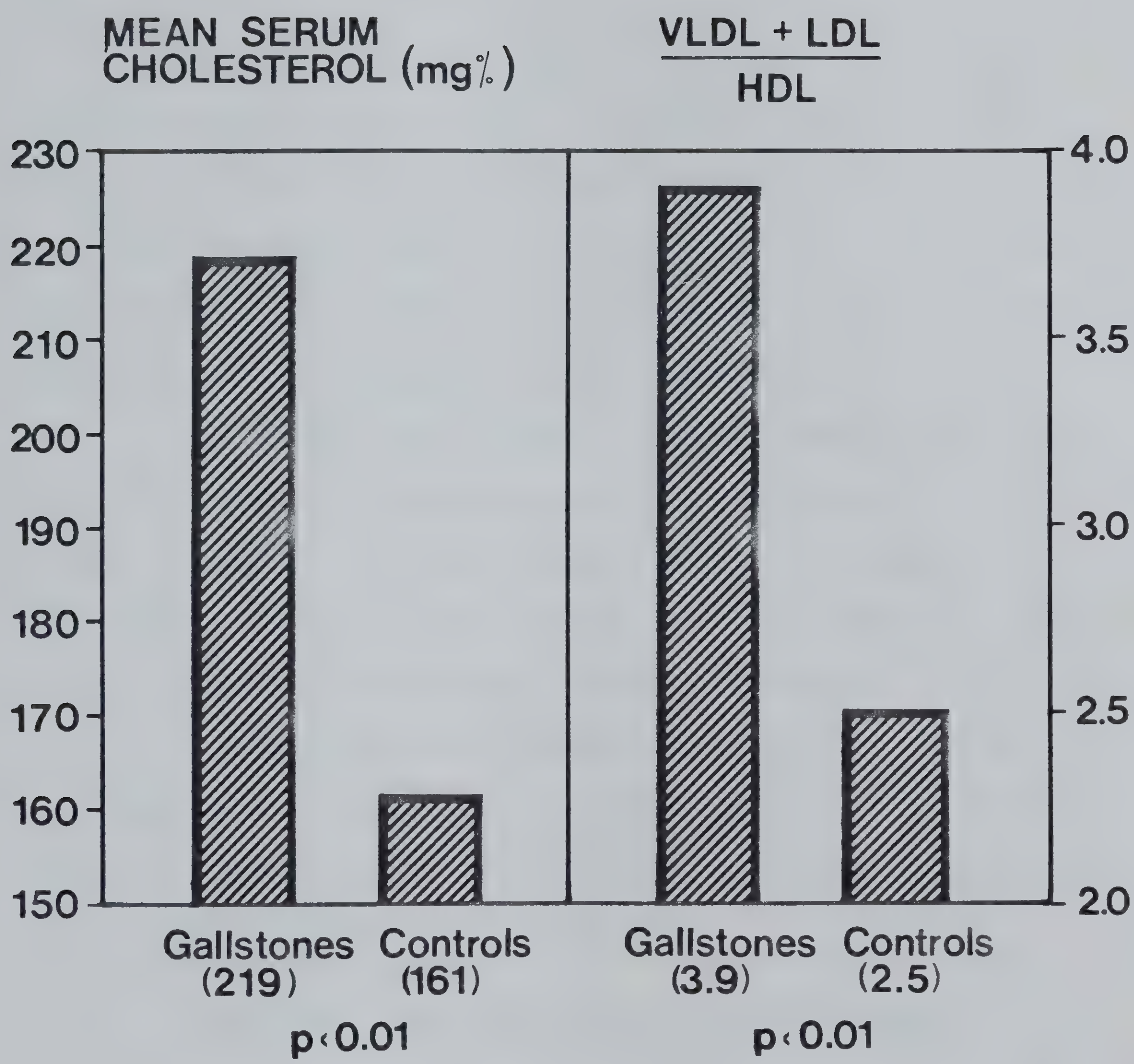


Figure 15. Mean Serum Cholesterol and Lipoprotein Ratios

112 ± 16.5 mg%. Triglycerides were elevated when compared to controls (195 ± 23.9 mg% $p < 0.01$) and did not change significantly with bran (219 ± 34.2 mg%).

One month on the bran diet did not change the lipoprotein cholesterol ratio (VLDL + LDL/HDL) in the control group (2.5 ± 0.31 versus 2.4 ± 0.28). The ratio was significantly elevated in the gallstone group when compared to controls (3.9 ± 0.39 $p < 0.01$). No change in the ratio was observed after one month of bran (3.8 ± 0.41). Lipoprotein data are presented in Table 10 and Figure 15.

Four patients were followed from 6 - 12 months on the bran diet. HDL cholesterol increased significantly from 43 mg% to 60 mg% ± SE 13.6, $p < 0.01$. (Fig. 16). Lipoprotein ratios decreased significantly from 4.3 to 2.3 ± SE 0.26, $p < 0.01$. (Fig. 16). Total serum triglycerides and cholesterol remained unchanged.

It is noteworthy that the bowel habit of every volunteer was changed by bran, usually from one bowel movement per day to two to three bulky, soft stools per day. No significant change in weight was observed during the one month bran diet.

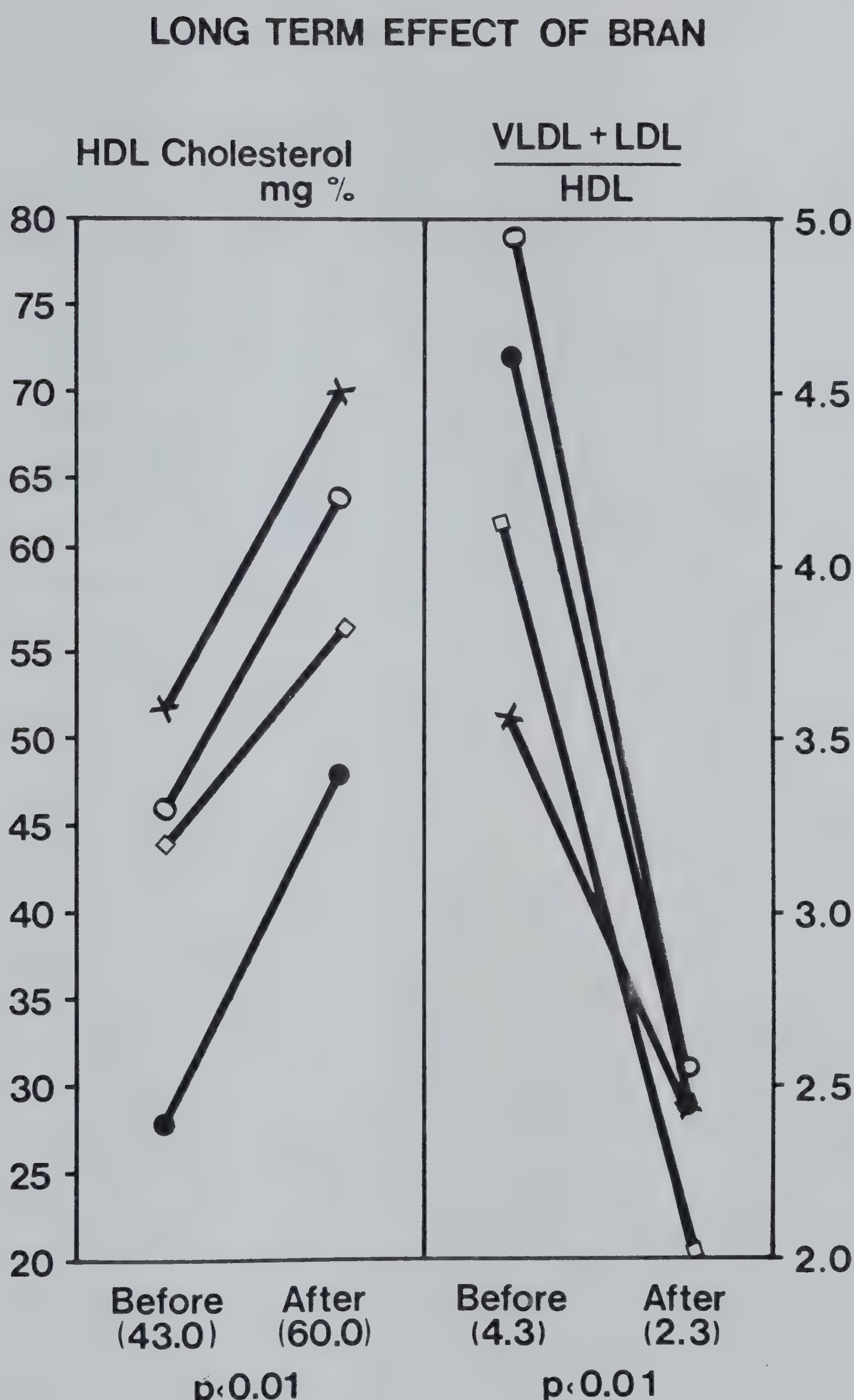
PART III: FECAL BILE ACIDS AND NEUTRAL STEROLS

The four patients in this investigation all had normal serum total protein, albumin, SGOT, alkaline phosphatase, LDH, and bilirubin throughout the duration of the experiment. Following bran, stool volume was felt to be increased by all patients and the three with constipation were "cured", having 1 - 3 soft, bulky bowel

TABLE 10

High Density Lipoproteins and Lipoprotein Ratios

PATIENT	HIGH DENSITY LIPOPROTEIN Before	After	CHOLESTEROL (mg%) RATIOS Before	After
Controls				
1. Oli	48	51	3.5	3.5
2. Rod	-	-	-	-
3. Jam	59	54	1.9	1.9
4. Gle	47	43	2.3	1.9
5. Joy	80	78	1.3	1.2
6. Hea	44	44	2.4	3.1
7. Lan	42	54	4.0	3.2
8. Ros	50	53	2.2	1.9
9. Bru	45	48	2.0	2.1
MEAN	52	53	2.5	2.4
Gallstones				
1. Dav	57	63	3.8	3.2
2. Koz	53	57	3.0	3.2
3. Bel	44	40	4.0	4.8
4. Ing	46	44	4.9	4.7
5. Yak	52	-	3.6	-
6. Kat	68	81	2.0	1.9
7. Pat	53	53	3.4	3.2
8. Gni	28	33	4.7	4.1
9. Tam	42	44	5.4	5.5
MEAN	49	52	3.9	3.8



LONG TERM EFFECT OF BRAN
HDL Cholesterol mg %
VLDL + LDL / HDL
80
75
70
65
60
55
50
45
40
35
30
25
20
5.0
4.5
4.0
3.5
3.0
2.5
2.0
Before (43.0)
After (60.0)
p<0.01
Before (4.3)
After (2.3)
p<0.01

Figure 16

movements per day.

Results of fecal bile acid neutral sterol and fecal weight determinations are presented in Table 11. Mean fecal weight showed a trend to increase, changing from 121 gm/day ± SD = 65 to 238 gm/day ± 19 $p < 0.10$. The daily mean bile acid excretion increased quite significantly from 0.28 gm/day ± 0.12 to 0.64 gm/day ± 0.12, $p < 0.01$. Neutral sterol excretion did not change significantly, although the mean excretion increased by over 70% (0.74 gm/day ± 0.25 versus 1.26 gm/day ± 0.33). Total sterol excretion, bile acids plus neutral sterols increased significantly from 1.02 gm/day ± 0.33 to 1.90 gm/day ± 0.43, $p < 0.05$.

TABLE 11

Fecal Data

PATIENT	STOOL WT (gm/day)		BILE ACIDS (gm/day)		NEUTRAL STEROLS (gm/day)	
	Before	After	Before	After	Before	After
1. Ock	63	216	0.39	0.87	1.13	2.21
2. Han	109	227	0.55	0.78	0.79	1.18
3. Mor	305	293	0.16	0.59	1.02	0.86
4. Pal	5.3	214	0.025	0.31	0.023	0.78
MEAN	121	238	0.28	0.64	0.74	1.26

DISCUSSION

BILE ACID FRACTIONATION

In this investigation, bile was obtained by aspiration of the gallbladder at the time of surgery in anesthetized patients following at least a ten hour fast. All patients selected for study were undergoing elective surgery and had no history of medical problems. There was a preponderence of females in the gallstone group (due to the paucity of suitable males undergoing cholecystectomy at the time of this study) and the results must be interpreted with this in mind. Estrogens are able to increase the lithogenicity of bile, however there is little evidence that they affect the relative proportions of bile acids in bile.[1 34 35 36 316] Males are quite capable of forming gallstones and although estrogens can increase the tendency to form gallstones, they are nonetheless rare in rural African females, according to Burkitt.[1 13 36 37] Such observations indicate that investigators researching the etiology of cholesterol gallstones need not be over-concerned by the sex ratio of the disease, since estrogens per se are not the cause of gallstones. Mean body weight in the gallstone group was slightly higher than controls; however, small differences in weight should not affect the bile acid composition of the bile acid pool. The results from this study should be interpreted bearing in mind that the groups are not well matched.

Only gallbladder bile obtained from fasting subjects was

used in any of the investigations. Metzger has shown that fasting hepatic bile is more lithogenic than bile obtained at other times.[77] During fasting, much of the bile acid pool is stored in the gallbladder. Under these conditions only part of the pool will be available for recirculation, and there will be a reduction in bile acid secretion, *de novo* synthesis being slow to compensate.[250] Consequently, fasting hepatic bile will become more lithogenic due to reduced bile salt return to the liver. Decreased return of CDCA may result in release of HMG-CoA reductase from inhibition and add to the relative increase of cholesterol in fasting bile.[283] On the other hand, gallbladder bile obtained during fasting is said to be representative of average daily bile composition. After an overnight fast several hours of pooled bile secretion by the liver are available for sampling. In the same subject, hepatic and gallbladder bile composition are similar, although gallbladder bile is more concentrated and somewhat less lithogenic.[79 80] In this context, duodenal bile obtained after stimulated contraction of the gallbladder is also representative of average daily bile composition. Vlahcevic has compared gallbladder and duodenal aspirates in the same patients and found them to be quite similar, aside from concentration.[312]

Gallbladder bile specimens were cultured to exclude infection, since inflammation of the gallbladder can result in the absorption of bile acids.[25 26] Similarly, infection can result in the deconjugation of bilirubin glucuronide and consequently the precipitation of calcium bilirubinate, which can form a nucleus for cholesterol precipitation.[8]

Serum determinations of total proteins, albumen, SGOT, LDH, alkaline phosphatase and bilirubin were obtained to exclude the possibility of liver disease. Significant liver disease results in impaired hepatic synthesis and clearance of many important compounds, as illustrated by cirrhotics or patients with biliary tract obstruction, and can influence the composition of bile.[64] Bile samples were checked and found to be within the 3 - 25% solids range, as recommended by Small for applicability to his line of cholesterol solubility.[5]

Analyses of total bile acids, phospholipids and cholesterol were obtained using precise standard reference methods in duplicate. Lack of agreement between duplicates of greater than 4% resulted in a repeat test, which was rare. Bile acid fractionation was judged to be accurate and complete by adding pure conjugated and unconjugated bile acids to both water and bile specimens in each run. Recoveries for each bile acid below 90%, or above 110%, were regarded as unacceptable and resulted in a rare repeat test. Similarly, duplicate samples were required to agree to within 5% to ensure precision of the analysis.

The finding of crystals in gallstone bile but not in control subjects confirms previous observations.[313] Similarly, there is clear separation of bile from gallstone patients and from control subjects on the basis of cholesterol supersaturation.[5] Analysis for cholesterol content of the gallstones confirmed that the gallstone group had cholesterol stones. This information clearly demonstrates that we are comparing a group of patients with cholesterol gallstones,

lithogenic bile and crystals, to a group without stones and who have unsaturated bile.

The finding of increased DCA in the bile of gallstone patients fails to confirm the findings of Pomare and Heaton, in which there was no significant difference in DCA between gallstone patients and controls, although a trend was shown.[314] The difference may be due to methodology, since they used thin-layer chromatography for bile acid fractionation, and this technique does not have the accuracy or sensitivity of gas-liquid chromatography (GLC). Several groups of investigators have observed an increase in DCA in gallstone bile but failed to comment on this finding.[186 188 315 316]

Our investigation failed to confirm the finding of decreased CDCA in gallstone patients, an observation that is well supported.[183 190] This discrepancy is probably due to the small number of patients used in this study and would disappear with the addition of more patients. Furthermore, in the Bran Diet experiment, where more people were studied, a decrease of CDCA was observed in gallstone patients.

An explanation for the observed increase in DCA may be found in considering the enterohepatic circulation (EHC) of bile acids. Since DCA is formed by bacterial 7α-dehydroxylation of CA in the colon, any factor which could increase the rate of this reaction or the absorption of DCA will explain this finding.[105] Possible explanations are:

1. Alterations in bacterial flora, producing 7α-dehydroxylase.

2. Increased intestinal transit time (colonic stasis), which could allow longer contact of CA with bacterial enzymes, and more time for absorption of DCA.
3. Lack of bile acid binding substances in the diet such as fiber, which would result in decreased excretion of DCA, and perhaps increased exposure of CA to bacterial enzymes.[317 318]
4. Absence or reduction of the gallbladder reservoir or increased contractility due to the presence of stones, could result in increased contact of the bile acid pool with the gut, and consequently increase DCA production.[184]

DCA has been suggested by Low-Beer to be responsible for the formation of gallstones.[191] This hypothesis is based on the observation that DCA selectively inhibited the synthesis of CDCA in three patients.[245] CDCA is known to desaturate bile by its effects on HMG-CoA reductase.[283] Feeding DCA to healthy volunteers increased the cholesterol saturation of their bile.[245] Further work by members of the same research group demonstrated feeding bran produced a reduction of DCA, an increase in CDCA, and reduced saturation of bile.[319 320] This information, when considered in light of Burkitt's observations on the epidemiology of cholesterol gallstones relating their scarcity in rural Africa to a high fiber diet, led us to investigate the effects of a bran diet on biliary lipid metabolism.[13]

BRAN DIET

In this experiment, biliary lipid metabolism was investigated in nine gallstone patients and nine control volunteers before and

after four weeks of consuming 50 gm of All Bran daily in addition to their regular diets. Although the mean body weight in both groups was similar, the gallstone group was much older than the controls. Sex ratios were also different, with only one male in the gallstone group and only two females in the control group. It is perhaps unfortunate that there were so many females in the gallstone group, however we were dependent upon referral of gallstone patients and had no control over sex ratios. The sex and age of the control group was purposely selected to be young and predominantly male. By confining this group to a young, healthy, fit and predominantly male population, we hoped to avoid individuals in a pre-gallstone state.[81] It was not, therefore, considered necessary to obtain oral cholecystograms in this group, thereby reducing the radiation exposure to healthy normal volunteers. Comparison of data between these groups, however, must be interpreted with the consideration that they are poorly sex matched and purposely age mis-matched. Careful preliminary interviews were conducted to ensure that all patients and volunteers in this study were well-motivated, co-operative, and well-informed about the requirements and procedures involved.

A profile of liver function (total proteins, albumin, SGOT, LDH, alkaline phosphatase, bilirubin) was obtained at the beginning and the end of the experiment to be sure that liver function was normal and remained normal throughout the investigation (which it did).

All bile specimens were obtained by duodenal intubation, and as discussed, duodenal bile obtained after gallbladder contraction

is representative of gallbladder bile.[312] 10% Travasol was used to stimulate gallbladder contraction since amino acids would not interfere with biliary lipid analysis and it is less expensive and potentially less hazardous than an intravenous injection of cholecystokinin. Fluoroscopic positioning of the enteroclysis tube was employed to ensure optimum placement of the tube. There were no failures to obtain a bile sample by this method.

Examination of bile for cholesterol crystals revealed none in the control group. Crystals were seen in several patients initially in the gallstone group, and disappeared from the post-bran specimens. Failure to observe crystals in all the initial gallstone bile specimens may be related to concentration. Crystals were never observed in dilute (<10 mM bile acids) specimens. The disappearance of crystals from these gallstone patients suggests that the bran diet had induced desaturation of their bile.

Biliary lipid analysis methodology was somewhat different than in the previous study. Since that time we were able to quantitate cholesterol and bile acids simultaneously by GLC. Furthermore, on dilute specimens, GLC was found to be more accurate than the standard reference methods (closer agreement of duplicates, "no-nonsense" results). However, the standard reference methods were still used as a check. In rare cases of marked disagreement between methods, the sample was re-analyzed by both methods, and the reliability of GLC for dilute samples was usually confirmed.

Small's line of cholesterol solubility was again used to

determine degree of cholesterol saturation.[5] Small and Carey have both investigated and discussed the importance of concentration (percent solids) on the solubility of cholesterol in bile.[321 322] For example, below 5 gm/100 ml the solubility decreases abruptly as total concentration of lipid is reduced.[322] Although the duodenal aspirates were occasionally quite dilute, the bile was obtained by stimulated gallbladder contraction and should represent gallbladder bile. Since there was no way of determining original concentration of the gallbladder bile, we had to assume that the percent solids were within a range acceptable for application of Small's solubility line.[5]

Our demonstration of the ability of 50 gm daily of All Bran to desaturate bile in gallstone patients and its tendency to reduce saturation in control subjects is in agreement with the Bristol group (Pomare, Low-Beer and Heaton) and also Watts et al in Australia.[320 323] Only the Bristol group studied the effect of bran in gallstone patients (10) and the reduction in saturation was less (mean lithogenic index 1.49 fell to 1.29). The dose of bran ranged from 20 - 108 gm daily, averaging 57 gm, which may explain the discrepancy in results. Total bile acids were measured by an enzymatic method, as compared to our use of GLC.[320] As discussed, GLC is more accurate for determining total bile acids in dilute duodenal aspirates. Perhaps if the amount of bran used and methodologies employed were standardized, the magnitude of the results would be similar.

The ability of bran to desaturate bile confirms Burkitt's

hypothesis that cholesterol cholelithiasis is a fiber deficiency disease.[13] It seems more than likely that if a high risk population, such as Canadians, were to increase their daily intake of fiber lithogenic bile and its sequelae (gallstones) would become less common. The desaturation of bile suggests that as a longterm possibility a high fiber diet may induce gallstone dissolution, since the changes in saturation are similar to those seen with CDCA therapy.[190 268 269 275 276] Additional dietary fiber may be helpful following gallstone dissolution with CDCA. A major drawback to CDCA therapy is the prompt return of lithogenic bile and the reappearance of stones following its withdrawal.[271 272 273 274] Following dissolution, the addition of dietary fiber could prevent the return of cholesterol supersaturation and the reappearance of gallstones.

Although the diets of the people in this study were not quantitated or controlled (except for the bran), all persons stated that their diet did not change during the period of the experiment. Furthermore, weight did not change over the four week period, a fact which assumes some importance considering the relationship of obesity and over-nutrition to the saturation of bile and presence of gallstones.[1 31 38 39 40 41 42]

Pool size was determined by the technique of isotope dilution. An intravenous injection of an ethanolic solution containing 4 μCi of carboxyl ^{14}C cholic acid was administered 14½ hours prior to obtaining the bile specimen. The intravenous route was used in preference to the oral route to avoid the uncertainty of absorption

if given orally.[115] Isotopic 24-^{14}C cholic acid was chosen for several reasons. ^{14}C is conveniently and easily counted, and although bile acids labelled on the amino acid conjugates are available, pool size measurements using these labelled bile acids are invalid because of bacterial deconjugation during enterohepatic cycling. Tritium labelled bile acids were not used because the label tends to be lost during intestinal passage.[324] A 4μCi dose was chosen because it was calculated that the gallbladder mucosa, assumed to be the organ at risk, receives 133 millirads per μCi ^{14}C.[325] This dose would result in a total exposure of less than 1 rad per volunteer and still be compatible with accurate counting of the bile samples. This effort to keep isotope dose to a minimum assumes even greater importance in view of the fact that abdominal fluoroscopy (during tube positioning) results in a radiation exposure of about 400 millirads per minute.[325]

Unfortunately (fortunately for the volunteers) the use of such a low dose of isotope precluded the use of the classical Lindstedt procedure involving intubations for 4 - 5 consecutive days, since the activity of the bile samples would be too low to count accurately after a few days.[113] Therefore, we were unable to obtain any data concerning turnover and synthetic rates.[115] The use of a single intubation has been used and compared to the Lindstedt technique by two groups.[306, 326] Precision on repeat determinations was excellent, and comparison to Lindstedt pools showed only slightly higher estimates.[306, 326] Our 14½ hour determination should therefore be precise, accurate and reproducable, although strictly speaking not directly comparable to the pool size estimates obtained by other

investigators. Keeping the time variation between injection to sampling to ± ½ hour permits valid comparison of pool sizes within our series.

Decolorization of all samples with H_2O_2, prior to liquid scintillation counting, eliminated the problem of color quenching, and permitted valid comparison of pool size estimates obtained from dilute and concentrated samples.[307 327] Ascorbic acid was added following decolorization to scavenge excess H_2O_2 which might have caused chemiluminescence owing to the liberated oxygen.[327]

Total pool size was found to be reduced in the gallstone patients, confirming the observations of many other investigators.[183 185 188 195] Pool size showed a trend to increase in gallstone patients, but not controls, following bran. This measurement was not made during the Bristol group's experiments with gallstone patients.[320] Failure of the pool size to change in control patients was observed in the Bristol study, however the Australian group did not measure pool size.[320 323]

The trend for pool size to increase with bran intake in gallstone patients may be explained by two possibilities. Bran has been shown to bind bile acids.[317 318] This effect would tend to decrease the number of daily enterohepatic circulations the bile acid pool makes, and therefore pool size would expand (pool size x circulations = constant).[195 196] Another possibility is that fiber will bind the isotopic CA causing the activity of the bile specimens to be reduced, and the pool to appear larger.

The trend to increase pool size does not correlate well with the obvious decrease in cholesterol saturation and is not likely to be responsible for the improvement. In fact, gallstone patients #4 and #7 (Table 8) had a decrease in pool size in spite of the improvement in their lithogenic index. Desaturation of bile without a significant increase in pool size has also been demonstrated during CDCA therapy.[269, 276, 281]

Decreased pool size, as opposed to the suggestions of Vlahcevic, may be the result rather than the cause of gallstones.[183, 185] We observed changes in pool sizes in ten dogs before, and one month after, implanting one or more spherical prostheses of glass and/or silastic in the gallbladder lumen through a cholecystostomy incision. These results were compared to pool sizes in five dogs before, and one month after, a sham operation in which a cholecystostomy incision was made, but no prosthetic gallstones were implanted. Decrease in pool size in the implant group was a highly significant average of 29.5%. The sham operated group had a less significant decrease of 8.2%.[328] It is strongly suggested by observations in this investigation, and by pool size results from the bran study, that a reduced pool per se is not responsible for the formation of lithogenic bile. The observed decreased pools in gallstone patients may be secondary to bile displacement by stones and/or interference with gallbladder distensibility or contractility due to their presence.

Bile acid fractionation was performed on all bile specimens

before and after the bran diet. Again, DCA was found to be significantly increased in gallstone patients; furthermore, with bran DCA showed a tendency to decrease in the control patients and decreased quite significantly in the gallstone group. These findings are in agreement with the Bristol group, however the Australian group (who used only 30 gm of bran) saw no consistent decrease in DCA following bran.[319 320 323] CDCA was significantly lower in gallstone patients, an observation consistent with other investigators.[183 190] CA was similar in both controls and gallstone patients and showed no consistent change following bran. Similarly, the proportion of CDCA in the bile acid pool showed no significant change. The Bristol group observed an increase of CDCA following bran, but this may be questioned on the basis of the small number of patients (six: two stone, four: normal) and the methodology (thin-layer chromatography) used in their investigation.[320]

Reduction of DCA by increased dietary fiber is a result of the known effects of bran to bind bile acids and to decrease intestinal transit time.[317 318 329] Binding and reduced time for bacterial dehydroxylation of CA and for absorption of DCA are responsible for decreased DCA in bile. Although there was no significant increase in CA or CDCA, the total primary bile acids increased to the same extent that DCA decreased. The observed reduction in DCA suggests that daily excretion of bile acids is increased with bran. This in turn would indicate an increased synthesis of primary bile acids since total pool size was maintained in spite of increased daily loss.

Failure of CDCA to increase in our investigation casts some doubt on the Bristol group's hypothesis that DCA inhibits CDCA synthesis, which in turn results in increased cholesterol synthesis, producing lithogenic bile and gallstones.[191 245 319 320] In support of our findings a sophisticated investigation by LaRusso, using GLC, demonstrated that DCA feeding did not increase cholesterol saturation in bile and specifically did not inhibit CDCA synthesis in seven volunteers.[330] Furthermore, Einarsson failed to observe consistent inhibition of CDCA synthesis during DCA feeding in six volunteers. Unfortunately, biliary cholesterol was not determined.[331]

Since desaturation of bile by bran does not seem to be produced by changes in pool size or reduction in DCA, it must be related to increased daily excretion of bile acids. Because DCA was reduced while pool size tended to increase, it may be inferred that daily bile acid excretion was increased by the bran diet with a consequent increase in primary bile acid synthesis. A similar mechanism has been elucidated by Strasberg. He has shown that in the Rhesus monkey during chronic partial (7.5%) controlled interruption of the enterohepatic circulation (EHC) using a bile fistula, cholesterol secretion decreases as bile acid synthesis increases.[158 242] By increasing bile acid excretion, bran is similarly producing a partial interruption of the EHC, and thereby decreasing cholesterol secretion and saturation. Reduction in cholesterol secretion has three possible mechanisms in this model. (Fig. 17)

1. Diversion of cholesterol into bile acid synthesis to compensate

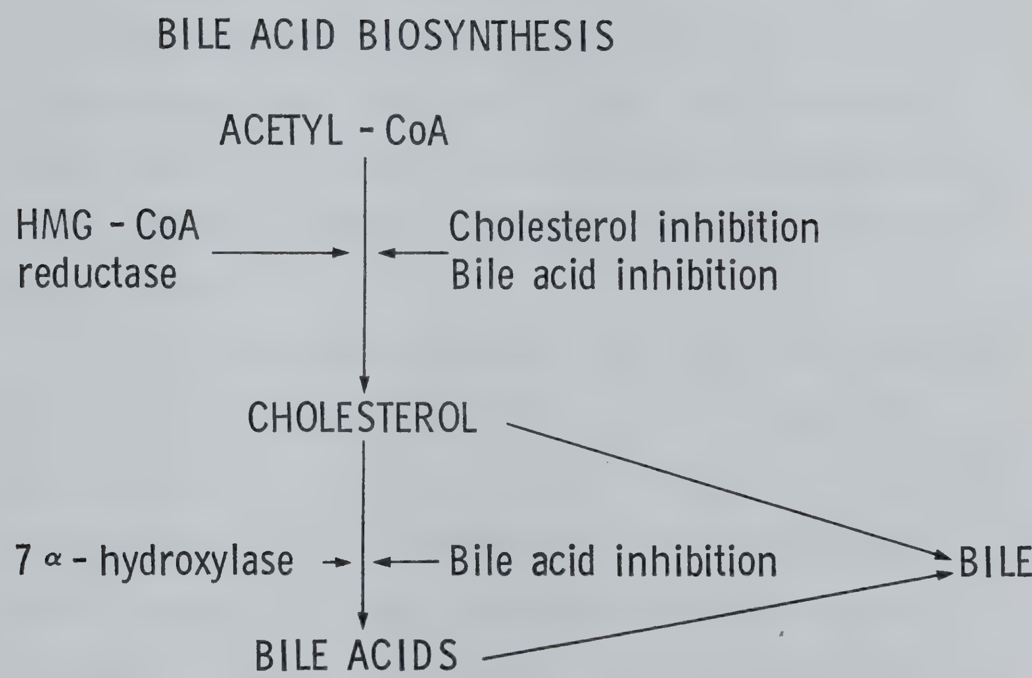

BILE ACID BIOSYNTHESIS
ACETYL - CoA
HMG - CoA reductase
Cholesterol inhibition
Bile acid inhibition
CHOLESTEROL
7 α - hydroxylase
Bile acid inhibition
BILE
BILE ACIDS

Figure 17

for increased bile acid excretion.[117]

2. Increased cholesterol excretion - bile acid binding will interfere with cholesterol absorption.[142]

3. Increased CDCA synthesis may inhibit HMG-CoA reductase, and therefore cholesterol secretion.[276]

FECAL BILE ACIDS AND NEUTRAL STEROLS

To quantitate the effects of the 50 gm All Bran diet on bile acid and neutral sterol excretion, 72 hour stool collections were obtained from four volunteers before and after four weeks on the diet. Results from this investigation confirm that sterol excretion is increased by bran.

Although the mean daily excretion of feces nearly doubled, this was not quite statistically significant. The discrepancy is explained by the fact that volunteer #3 (Table 11) had a very high fecal output initially (possibly related to cloxacillin therapy) which did not increase with bran, and consequently the calculated "P" value was greatly reduced. Transit time in this individual was initially so short that bran was apparently unable to cause a further decrease, and subsequent increase in fecal volume.

Mean daily bile acid excretion more than doubled, and this increase was highly significant ($p < 0.01$). Even though daily stool weight decreased in volunteer #3, her daily bile acid excretion increased from 160 mg to 590 mg, demonstrating the ability of bran to increase bile acid excretion without changing fecal volume. This observation supports the *in vitro* observations that fiber binds bile

acids.[317, 318] Although mean total neutral sterol excretion increased by 67%, the observed decrease in neutral sterol in volunteer #3 caused the increase in neutral sterol excretion to appear not significant. An explanation for the decrease in neutral sterols in this volunteer is not apparent, however the slight decrease in stool weight in spite of her personal statement that bran increased the volume and frequency of her bowel movements, suggests that the 72 hour stool collection was not complete.

There is little question from the observations of other investigators that bran increases the bulk of stool and speeds intestinal transit.[329, 332, 333, 334, 335] Bile acid excretion was not found to increase in some investigations using bran, however total sterol excretion was increased.[333, 334, 335] It is generally accepted that fiber from various sources can increase bile acid excretion.[332, 336] The effects of fiber and bran on sterol excretion are somewhat confused by the different amounts, types of fiber, and different methodologies used in the growing numbers of studies on this subject.

Dietary fiber can be defined as plant material that is impervious to the degradative action of digestive enzymes or the enzymes of the intestinal microflora.[337] Most dietary fiber is derived from the cell walls of plants and consists of cellulose, hemicellulose, lignins, pectins and gums. The types of fiber depend on plant species, age of the plant, and portion of the plant used.[336] Lignin is by far the most active component of fiber for binding bile acids.[317] Hydrophobic bonding is the likely mechanism by which lignin (fiber) adsorbs

bile acids since adsorption is the greatest when ionization of the phenolic hydroxyls of lignin's phenylpropane units is suppressed by increasing acidity.[318] Dihydroxy bile acids are more strongly adsorbed than trihydroxy bile acids, and unconjugated bile acids are less strongly bound than free bile acids.[317] Incorporation of monoglycerides and fatty acids into the bile acid micelle reduces adsorption. Adsorption is adversely affected by short chain length, unsaturation and high concentration of fatty acid in the micelle.[338] Binding studies using different vegetable fibers have revealed a wide range in their ability to adsorb bile acids. Pure lignin is the best, followed by alfalfa, bran, maize meal, corn and sugar beet pulp.[318 337 339 340] The cationic adsorptive properties of fibers may interfere with electrolyte and mineral absorption.[336] Increased zinc excretion has been observed on a high fiber diet, however an opposing study found no difference in zinc or copper balance.[341 342] Several studies indicate that fiber may increase the losses of iron, calcium, magnesium, phosphorus, sodium and potassium.[332 341 342 343 344 345]

SERUM TRIGLYCERIDES AND CHOLESTEROL

Serum triglycerides, cholesterol, and the lipoprotein distribution were investigated during the bran diet study. Since we were interfering with bile acid and cholesterol excretion, perhaps there would be an effect on serum lipids as with cholestyramine therapy.[142] Furthermore, the studies of Burkitt and of Trowell suggest that there may be a relationship between fiber deficiency, hypercholesterolemia and atherosclerosis.[13 346]

In our study there was no change in mean serum triglycerides or cholesterol after one month on bran. This observation is consistent with the observations of most other investigators who have studied this problem.[347 348 349 350]

Mean serum triglycerides were elevated in the gallstone group, and again this observation is consistent with those of other investigators.[351 352] Although the mean serum cholesterol levels were significantly elevated (mean difference 58 mg%) compared to controls, this observation has not been confirmed. The young age of the control group is perhaps part of the reason for the observed difference.

Hypertriglyceridemia in gallstone patients suggests overnutrition since it is well known that carbohydrates induce triglyceride synthesis.[353] In view of Burkitt's hypothesis that cholesterol cholelithiasis is a fiber deficiency disease, and our biochemical confirmation of this theory, hypertriglyceridemia in gallstone patients assumes some relevance.[13] The "Western diet" contains a high proportion of refined carbohydrate with the exclusion of fiber.[13 346] Hypertriglyceridemia in gallstone patients may be the result of a diet high in refined carbohydrates with the simultaneous exclusion of fiber.

The lipoprotein distribution of cholesterol differed between gallstone patients and controls. There was significantly more cholesterol in the VLDL + LDL fraction and less in the HDL fraction in gallstone patients. Consequently, the lipoprotein ratios were significantly higher in the gallstone group. Cholesterol fractionation has not been studied in gallstone patients by other investigators. Lower

levels of HDL cholesterol in gallstone patients suggests that there is reduced transport of systemic cholesterol into the liver in these patients.[227 228 229]

Following six months to one year on bran, an increase of HDL cholesterol and a decrease in lipoprotein ratios to control levels was noted. The ability of a bran diet to increase HDL cholesterol has been observed by other investigators, however our study is by far the longest follow-up reported.[354 355] Return of the lipoprotein ratio of cholesterol in gallstone patients to control levels indicates the initial observed differences are not a function of age, and perhaps the high serum cholesterol in gallstones is similarly not a function of age. As a longterm possibility, a bran diet may reduce serum cholesterol, since it acts in a manner similar to cholestyramine, although it is not nearly as effective in binding bile acids.[142] Low amounts of HDL cholesterol have been correlated to the development of atherosclerosis.[228] Therefore our findings suggest a possible metabolic link between cholesterol gallstones, fiber deficiency and atherosclerosis.

Additionally, the increase in HDL cholesterol suggests that increased bile acid and neutral sterol excretion stimulates transport of systemic cholesterol to the liver in an attempt to compensate for increased loss from the biliary system as bile acids and neutral sterols. Some support for this hypothesis is obtained from the observations of Rachmilewitz who has observed labelled HDL remnants in bile.[227] Furthermore, Schwartz et al have found, when using intravenous

injections of labelled mevalonic acid and ^{14}C-cholesterol in bile fistula patients, that an appreciable amount of cholesterol necessary for bile acid synthesis and cholesterol secretion is derived from plasma cholesterol.[356]

SUMMARY

In summary, our investigations have shown that cholesterol cholelithiasis is likely the consequence of fiber deficiency. Increased dietary fiber reduces the cholesterol saturation of bile by promoting the excretion of cholesterol and its metabolites, the bile acids. Addition of fiber to the diets of high risk populations should reduce the incidence of gallstones and in those afflicted may evantually promote their dissolution. The bran diet also favourably changes the lipoprotein distribution of cholesterol, which may indicate a possible role for bran in the prevention of atherosclerosis.

CONCLUSIONS

The conclusions from these investigations are:

1. Biliary DCA is increased in gallstone patients, indicating an altered colonic phase of the enterohepatic circulation of bile acids.
2. Biliary supersaturation with cholesterol is consistently observed in the fasting bile of gallstone patients, but is rarely seen in persons without gallstones.
3. There is a decreased total bile acid pool in gallstone patients.
4. CDCA pool is reduced in gallstone patients.
5. Serum cholesterol and especially triglycerides are elevated in the majority of gallstone patients.
6. There is a decrease in high density lipoprotein cholesterol and an increase in lipoprotein cholesterol ratios in gallstone patients.
7. Cholesterol saturation of bile is effectively reduced by a high bran diet in gallstone patients.
8. The DCA pool is reduced by a high bran diet, especially in gallstone patients.
9. Total bile acid pool, CDCA and CA pools are not significantly changed by a high bran diet.
10. Daily stool volume, fecal bile acid and neutral steroid excretion are increased by the addition of bran to the diet of volunteers without biliary tract disease.

11. High fiber (bran) diets reduce cholesterol in bile by partially interrupting the enterohepatic circulation. Increased excretion of bile acids and cholesterol reduces the availability of cholesterol for excretion into bile. Bile acid synthesis occurs at an accelerated rate due to the increased daily loss and further reduces the availability of cholesterol for biliary secretion.
12. The longterm effect of bran on systemic cholesterol transport is to increase high density lipoprotein cholesterol and to reduce the lipoprotein cholesterol ratios. This effect is presumably due to increased transport of cholesterol to the liver to compensate for the increased metabolism and excretion of cholesterol.
13. Addition of fiber (bran) to populations predisposed to cholesterol gallstones may reduce the incidence of gallstones by reducing the amount of cholesterol in bile, relative to bile acids and phospholipids.
14. Bran may have some potential in the treatment of gallstones. The demonstrated reduction in cholesterol saturation could, as a longterm possibility, result in the dissolution of gallstones.
15. Following gallstone dissolution by CDCA or UDCA, bran may provide reasonable maintenance therapy to prevent the recurrence of stones.
16. Dietary fiber deficiency is probably the major etiological factor in cholesterol cholelithiasis since it explains the epidemiological data, and explains the association with overnutrition and

hyperglyceridemia. Experiments with increasing dietary fiber in man entirely support this hypothesis.

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APPENDIX A

PHOSPHOLIPID ANALYSIS

1. 1 ml specimen was added drop by drop to 22 ml $CHCl_3$ - CH_3OH (2:1) on the vortex and left to stand in excess of five minutes.
2. The tube was stoppered, shaken for 30 seconds, brought to 25 ml by adding more $CHCl_3$ - CH_3OH, and left to stand for an additional five minutes.
3. 5 ml dilute H_2SO_4 (1 ml concentrated H_2SO_4 to 2 liters deionized water) was added, the tube inverted 10 times, and left standing for 10 minutes.
4. Centrifugation at 2,000 RPM for 15 minutes separated the lower chloroform phase containing the lipids. Phosphorus was then liberated from the lipids and reacted with acid molybdate solution to form phosphomolybdic acid which was reduced by aminonaptholsulfonic acid to yield a blue colour by the following method:
    1. 5 ml extract was evaporated to dryness, 2.5 ml of 5N H_2SO_4 added, and the mixture slow boiled.
    2. After a black or brown colour change occurred, one drop of 30% H_2O_2 was added and heating continued for at least 10 minutes until the contents became colourless. If unsuccessful this step was repeated.
    3. A standard was prepared by transferring 0.5 ml of phosphate

standard (0.08 mg phosphorus per ml) to a digestion tube and adding 2.5 ml of 5N H_2SO_4. 2.5 ml of 5N H_2SO_4 was used as a blank. The same amount of H_2O_2 as used in step 2 was added, and the tubes boiled for 10 minutes.

4. Contents were diluted with a few ml deionized water, cooled to room temperature, and transferred to 25 ml volumetric flasks with repeated washings so the flask was half full.
5. 2.5 ml ammonium molybdate solution (2.5% w/v) and 1 ml aminonapthol-sulfonic acid reagent were added. Contents were diluted to the 25 ml mark with deionized water, mixed, and allowed to stand for five minutes.
6. Measurements of optical density were made with the Unicam S.P. 1800 spectrophotometer at 675 nm.
7. Calculations:

   $$\text{mg\% phospholipid} = \frac{\text{O.D. unknown}}{\text{O.D. standard}} \times 0.04 \times 18 \times 100 \times 25$$

   Millimolar concentration was found by employing the conversion factor:

   1 mM phospholipid = 793 mg/l.

APPENDIX B

CHOLESTEROL ANALYSIS

Reagents used were:

1. 95% ethyl alcohol, redistilled.
2. Petroleum ether B.P. 68°C, reagent grade.
3. Glacial acetic acid, reagent grade.
4. Concentrated H_2SO_4, reagent grade.
5. Acetic anhydride, reagent grade, free from HCl.
6. KOH solution 33% w/v.
7. Ethanolic KOH solution, prepared immediately before use (6 ml 33% KOH to 94 ml 95% ethyl alcohol).
8. Standard cholesterol solution (100 mg cholesterol to absolute CH_3OH to make 250 ml).
9. Modified Liebermann-Burchard reagent (1 vol. concentrated H_2SO_4 to 20 vol. acetic anhydride, chilled to less than 10°C, kept cold for nine minutes, and 10 vol. glacial acetic acid added).

The procedure was:

1. Standards - 0.3 ml of 33% KOH was added to a 25 or 50 ml glass stoppered centrifuge tube containing 5 ml cholesterol standard.
2. 0.5 ml bile were transferred to centrifuge tubes and 5 ml alcoholic KOH added. Tubes were stoppered, shaken well, and

placed in a water bath at 37 - 40°C for 55 minutes.

3. After cooling to room temperature, 10 ml of petroleum ether was added and mixed well, 5 ml water added and shaken vigorously for one minute, and centrifuged for 10 minutes.
4. Test: Duplicate 4 ml aliquots of the petroleum ether layer were transferred to large dry test tubes. Standards: duplicate aliquots, 1.0, 2.0 and 3.0 ml (equivalent to 0.2, 0.4 and 0.6 mg cholesterol) were transferred to similar test tubes. All tubes were evaporated to dryness at 60°C in a water bath while blowing a gentle stream of nitrogen into them.
5. After cooling, test tubes were arranged so that one set of standards was at the beginning and the other at the end of the series, and a clean empty test tube was placed at the beginning to receive the blank.
6. 6 ml of modified Liebermann-Burchard reagent was added, beginning with the blank, and at one minute intervals, to the samples. Tubes were stoppered, mixed using a shaker, and optical densities read at 620 mμ exactly 30 minutes after addition of the reagent.
7. Calculations:

$$\text{Optical density equivalent to 1 mg cholesterol (S)} = \frac{\text{O.D. standard}}{\text{mg cholesterol in aliquot}}$$

$$\text{mg \% cholesterol} = \frac{\text{O.D. unknown}}{\text{S}} \times \frac{10}{4} \times \frac{100}{0.5}$$

Millimolar concentration of cholesterol was calculated using the conversion factor: 1 mM = 387 mg/1.

APPENDIX C

TOTAL BILE ACIDS METHOD

Reagents were:

1. Buffer, 0.1 M sodium pyrophosphate.
2. Hydrazine sulphate 1.0 M (3.4 ml 95% hydrazine plus 1.5 ml concentrated H_2SO_4, q.s. to 100 ml with deionized water).
3. NAD, 6.8 mM.
4. Enzyme prepared from Pseudomonas testosteroni (Sigma, Type 1).

Procedure was carried out as per Table 12. All tubes were then incubated at 37°C for 60 minutes. Spectrophotometric determinations were made at 340 nm. A graph was constructed by plotting mM concentration of chenodeoxycholic acid standards against optical density and values obtained for the bile samples from their optical densities.

TABLE 12

Bile Salt Analysis Procedure: Assay

	Blank (ml)	Test (ml)	CDCA	Standards	ml
Buffer	2.0	2.0	2.0	2.0	2.0
Hydrazine	1.0	1.0	1.0	1.0	1.0
NAD	0.5	0.5	0.5	0.5	0.5
Bile	0.003	0.003	-	-	-
Enzyme	-	0.025	0.025	0.025	0.025
Boiled Enzyme	0.025	-	-	-	-
Chenodeoxycholate Acid	-	-	0.01	0.03	0.05

APPENDIX D

BILE ACID FRACTIONATION (GLC)

Preparation of Samples (To be done in Duplicate)

Standards

1. To three 15 ml capped test tubes add 50, 100, and 200 μl of an ethanolic solution containing 0.5 mg/ml of lithocholic, deoxycholic, chenodeoxycholic and cholic acids.
2. Similarly add 50, 100, and 200 μl of a 0.4 mg/ml methanolic solution of cholesterol.
3. Add 100 μl of a 2 mg/ml ethanolic solution of 7-ketodeoxycholic acid as an internal standard.

Conjugated Standards

1. To a single capped test tube add 100 μl each of a 2 mg/ml solution of glycocholic and taurochenodeoxycholic acids.
2. Evaporate to dryness in a 60°C water bath, blowing with nitrogen.
3. Add 1 ml sodium acetate buffer pH 5.6, 0.1 M.

Specimens - Recoveries

1. Dilute specimen by adding 100 μl to 3 ml acetate buffer.
2. Add 1 ml dilute bile to three capped test tubes #1, 2, and 3.
3. Add 100 μl each of the 2 mg/ml ethanolic solutions of glycocholic and taurochenodeoxycholic acids to tube #2.

4. Add 100 μl cholesterol to tube #3.

Specimens - Additional

Prepare dilute bile as above and add 1 ml to a single capped test tube. Repeat for as many samples as necessary.

Hydrolysis

Preparation of Enzyme

1. For each specimen plus the conjugated standards, grind 10 mg Clostridium welchii acetone powder (containing cholylglycine hydrolase) with 10 mg alumina in a mortar. Add 4 mg disodium EDTA and dissolve in 1.5 ml acetate buffer. Add 0.15 ml of a 0.1 M solution of β-mercaptoethanol. Centrifuge at 2000 RPM for a few minutes, and retain supernatant.
2. Add 1.5 ml enzyme solution to tubes containing specimens and conjugated standards.
3. React in a 37°C water bath for 4 - 5 hours.
4. Prior to extraction add 200 μl of the 0.5 mg/ml solution of free bile acids to specimen recovery tube #3.

Extraction of Free Bile Acids

1. Acidify to pH 1 by adding 1 ml 6N HCl.
2. Add 5 ml ether and run on shaker for 5 minutes. Centrifuge at 2000 RPM for one minute, and pipette supernatant into a capped test tube to which has been added 100 μl of a 2 mg/ml solution of 7-ketodeoxycholic acid. Repeat extraction three additional times.
3. Evaporate to dryness in 40°C water bath, blowing with nitrogen.

Methylation

To each test tube (standards and tests) add 1 ml of a 5:1 mixture of ether methanol.

Preparation of Diazomethane

1. Stopper two 50 ml test tubes and connect with glass tubing projecting 2 cm into the top of the reaction tube and projecting to the bottom of the collection tube. A second piece of tubing (N_2 inlet) goes through the stopper to the bottom of the reaction tube, and a third piece (N_2 outlet) projects 2 cm through the stopper of the collection tube.
2. Distillation apparatus is placed in a well ventilated fume hood and the collection tube is immersed in a beaker containing ice.
3. Add 15 ml ether to the collecting tube and 7 ml ether to the reaction tube.
4. Add 0.5 gm Diazold and 2 ml of alcoholic KOH (1.5 gm KOH/30 ml ethanol). Stopper tubes and bubble N_2 through, until reaction tube becomes whitish (20 minutes).
5. Add 1.5 ml diazomethane and react (10 minutes) until there is a persistent light yellow colour. Tightly cap tubes.
6. Place in a 60°C water bath and evaporate to dryness by blowing with N_2.

Preparation of TFA Derivatives

1. Add 0.3 ml trifluroacetic anhydride and tightly cap tubes. React at 37°C in a water bath for 30 minutes. Evaporate to dryness in a 60°C water bath by blowing with nitrogen.
2. Dissolve residue in 200 ml acetonitrile.

Gas-liquid Chromatography

Preparation of a Column

1. Silanization

   a. A glass column 1/8" x 3 ft. is washed with 50 ml of DIW, methanol, acetone, chloroform, and toluene, then silanized under a vacuum with 80 ml 2% dimethylchlorosilane in toluene (DMCS).

   b. Rinse with 200 ml methanol and leave under vacuum until dry, 6 hours.

   c. Glass wool. Soak in 1/200 photoflo/H_2O for three hours, blot dry, and dry at room temperature.

2. Packing

   a. Place a small plug of glass wool in the outlet and under vacuum pour small amounts of QF-1 (1.5% on chromasorb W AW-DMCS 80 - 100 mesh) into the inlet via funnel and tubing. Pack by tapping column with a pencil covered with rubber tubing. Fill to 5 cm below inlet.

   b. Protect inlet with a small plug of sialinized glass wool.

   c. Keep on vacuum overnight.

3. Column Conditioning

   a. Condition at 240°C (QF-1 temperature limit 250°) for a minimum of 48 hours, with the outlet disconnected and the carrier gas flowing at about half normal rate.

Analysis Conditions

Injector 280°C

Manifold 290°

Column 200° x 1 minute, increase by 16° per minute to 230°

Nitrogen 50 ml/min

Inject 1 μl samples.

Results - Calculations

1. Peaks from the four free bile acid standards will allow construction of a standard graph for each bile acid if desired. They can also be used to program an instrument such as the Hewlett-Packard 5830A GC to calculate the amounts of the individual bile acids in a sample.
2. Percent recovery: extraction can be determined for free bile acids and cholesterol from recovery specimens #1 and #3 amounts, i.e.,

$$\frac{\text{Bile + standard (\#3)} - \text{Bile (\#1)}}{\text{standard}} \times 100$$

3. Percent recovery: hydrolysis can similarly be determined using the results from the conjugated standard hydrolysis and the results from recovery specimens #1 and #2, i.e.,

$$\frac{\text{Bile + conjugated standard (\#2)} - \text{Bile (\#1)}}{\text{conjugated standard}} \times 100$$

4. If percent recoveries are acceptable, additional specimen results may be used without further verification if duplicates agree.

APPENDIX E

POOL SIZE DETERMINATION

Decolorization

1. Blank: add 0.5 ml deionized water, 0.5 ml tetramethylammonium hydroxide and 0.5 ml 30% H_2O_2 to a scintillation vial.
2. Isotope standard (C^{14} cholic acid): add 100, 200 and 300 μl isotope to three scintillation vials, q.s. deionized water to total volume of 0.5 ml. Add 0.5 ml tetramethylammonium hydroxide and 0.5 ml 30% H_2O_2.
3. Specimens: add 100, 200 and 300 μl of bile sample to three scintillation vials, q.s. deionized water to total volume of 0.5 ml. Add 0.5 ml tetramethylammonium hydroxide and 0.5 ml 30% H_2O_2.
4. React in a 65°C water bath until specimens have lost their color (usually about ten minutes).
5. Add 0.5 ml 3M Hcl and 0.5 ml ascorbic acid 15g/100 ml to all vials.
6. Add 15 ml Unogel Emulsifier (Schwarz Mann) to all vials and vortex until clear.

Scintillation Counting

Samples all counted in a Nuclear Chicago isocap/300 liquid scintillation counter for 10 minutes per sample, programmed for C^{14} external standard ratio.

Results

1. Quenching factor for all vials should be approximately the same.

2. Construct a graph volume vs. counts per minute for the isotope standards, extrapolate to 1 ml to give counts per ml for the ^{14}C cholic standard.
3. Construct a graph volume vs. counts per minute for the specimen dilutions and extrapolate to 1 ml to give counts per ml for the bile sample.
4. Pool size is calculated as follows:

$$\frac{\text{isotope standard cpm x ml isotope injected x mg bile acids/ml}}{\text{bile sample cpm x 1000}}$$

$$= \text{pool size (gm).}$$

APPENDIX F

STATISTICAL METHODS

The arithmetic mean and standard deviation were calculated by the following formulae:

Arithmetic Mean

$\bar{x}$ = mean

Σ = sum of

x = observation

N = number of observations

$$\bar{x} = \frac{\Sigma x}{N}$$

Standard Deviation

SD (standard deviation) = the square root of the arithmetic average of the squares of the differences between the observations and their mean.

$$SD = \frac{\Sigma(x - \bar{x})^2}{n - 1}$$

The unpaired Student's "t" test was used to determine whether the difference between the means of observations between two groups was significant. The paired Student's "t" test was used to determine the significance of the difference between the means of paired observations (observations before and after the supplementary dietary fiber).

Unpaired Student's "t" Test

$$t = \frac{\text{difference between both means}}{\text{standard error of the mean difference}}$$

$S\bar{d}$ (standard error) = expected variation occurring merely by chance in samples drawn randomly from the same group.

$$S\bar{d} = \sqrt{\frac{SD_1^{2} + SD_2^{2}}{N_1 + N_2}}$$

SD_1 = standard deviation of group 1

SD_2 = standard deviation of group 2

N_1 = number of observations in group 1

N_2 = number of observations in group 2

$$t = \frac{\bar{x}_1 - \bar{x}_2}{S\bar{d}}$$

$\bar{x}_1$ = mean of observations in group 1

$\bar{x}_2$ = mean of observations in group 2

Having calculated the "t" value, the probability value was obtained from the "t" table for the appropriate degrees of freedom (DF), which is the number of independent random selections that can be made from a sample.

$$DF = (N_1 + N_2) - 2$$

Paired Student's "t" Test

$$t = \frac{\text{difference between means of paired observations}}{\text{standard error of the mean difference}}$$

$$t = \frac{\bar{x}_1 - \bar{x}_2}{SE}$$

$\bar{x}_1$ = mean of observations before (bran)

$\bar{x}_2$ = mean of observations after (bran)

SE = standard error of mean difference between paired observations

$$SE = \sqrt{\frac{\Sigma\,(x_1 - x_2)^2 - \frac{(\Sigma\, x_1 - \Sigma\, x_2)^2}{n}}{n\,(n - 1)}}$$

The probability value was again obtained from the "t" table for the appropriate degrees of freedom.

APPENDIX G

DETERMINATION OF FECAL STEROIDS

1. Weigh and add 0.5 g of homogenized feces into a 50 ml glass stoppered centrifuge tube and add 6.0 ml 2 N NaOH.
2. Autoclave tubes at 120°C at 16 P. S. I. for 90 mintes.
3. After autoclaving add to each tube 20 ml ethyl acetate and shake manually for 30 seconds.
4. Add 2 ml concentrated HCl, mix by gentle agitation, allow to cool and shake mechanically for 10 minutes.
5. Centrifuge at 2,000 RPM for 5 minutes. Transfer upper layer to another 50 ml tube.
6. Perform a second extraction using 10 ml ethyl acetate and evaporate the combined upper phases to dryness at 65°C under a stream of nitrogen.
7. Dissolve residue in 1.0 ml propanol.

Extract is now ready for determination of total bile acids by the enzymatic method as in Appendix C, or for fractionation by GLC following derivatization as in Appendix D.